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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The oxidative stress hypothesis of Parkinson's disease (PD) is thought to involve the superoxide radical and nitric oxide (NO). It is believed that these two react with each other to produce peroxynitrite, a compound that damages cellular components in the nigrostriatal dopaminergic pathway of the brain. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) a meperidine analog that mimics most of the hallmarks of PD, is the drug of choice for investigations into the mechanism(s) of PD. Our studies indicate that of the three isoforms of the nitric oxide synthase (NOS) enzyme, both neuronal NOS (nNOS) and inducible NOS (iNOS) are up-regulated in the SNpc following MPTP administration due to increases in the nNOS and iNOS proteins. Also found was that MPTP had no effect on endothelial NOS (eNOS). During our studies, we noted a significant glial response in the SNpc of mice following MPTP treatments. We showed that microglia are a primary source of nitric oxide (NO). Also noted, was that microglia are a major source of the superoxide radical through the activation of NADPH oxidase. In our studies, we demonstrated several markers of both the oxidative modification and the nitration of key cellular components of the nigrostriatal dopaminergic in the MPTP mouse model and in postmortem PD tissues.

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ABSTRACT

This four year grant proposal was submitted to pursue studies on the pathogenesis of Parkinson's disease (PD) through the investigation of the molecular mechanisms of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that damages the nigrostriatal dopamine (DA) pathway as seen in PD. The PI's early research suggests that both the superoxide radical and nitric oxide (NO) contribute to MPTP toxicity. In determining the contribution of superoxide, NO, or both to MPTP toxicity, Specific Aim **SAI** compares the effects of MPTP on the nigrostriatal dopaminergic pathway of mice genetically engineered to express increased levels of copper/zinc superoxide dismutase activity (SOD1) and mice that express no neuronal NO synthase (nNOS-ko). Also to be explored are the crosses between these two lines of mice. MPTP upregulates inducible NOS (iNOS), another NOS isoform in the brain. This form of NOS seems to be part of a response to injury. Thus, to elucidate the role of iNOS in MPTP dopaminergic toxicity, **SAII** (1) characterizes the upregulation of iNOS following MPTP administration in wild-type mice, and (2) assesses the effects of MPTP on the nigrostriatal DA pathway of mutant mice deficient in iNOS. Both superoxide and NO are only modestly reactive, however, they can combine to produce the highly reactive tissue-damaging peroxynitrite. To demonstrate the production of peroxynitrite in the brain following MPTP administration, **SAIII** quantifies brain levels of dityrosine and 3-nitrotyrosine, the two main markers of the deleterious effects of peroxynitrite, on brain proteins, at different doses of MPTP and at different timepoints. Furthermore, to examine the biological consequences of nitration, **SAIV** assesses the nitration of candidate proteins such as mitochondrial electron transport chain polypeptides and manganese SOD in PC-12 cells exposed to peroxynitrite and MPP+, MPTP's active metabolite as well as in mice treated with MPTP. The catalytic activities of these enzymes are also examined. This proposal contains a comprehensive set of experiments that should provide important insights into the roles of superoxide and NO in MPTP toxicity and shed light on the mechanisms involved in neurodegeneration in PD.

INTRODUCTION

Parkinson's Disease is a common progressive neurodegenerative disorder characterized mainly by resting tremor, slowness of movement, rigidity and postural instability (1), all the result of a severe loss of dopamine (DA) neuron numbers in the substantia nigra pars compacta (SNpc) and a dramatic decrease in the number of dopaminergic terminals in the caudate-putamen (2). The prevelance of PD in North America alone is estimated at about 1,000,000 individuals with 50,000 newly diagnosed cases each year (1). The most potent treatment for PD still remains the administration of a precursor of DA, levodopa (L-DOPA), which replenishes the brain in DA, thus relieving almost all PD symptoms. However, the chronic administration of L-DOPA brings its own set of baggage as it often causes motor and psychiatric side effects which can be as debilitating as PD itself (3). Therefore, without undermining the importance of L-DOPA for the control of PD symptoms, it is urgent that we acquire a better and deeper understanding of the cause(s) of PD not only to prevent the disease but also to develop therapies aimed at halting the progression of the disease particularly in those newly diagnosed patients who may not require the use of L-DOPA during the early phases of PD.

Of the varied theories as to the causes of PD, the oxidative stress hypothesis is, by far, the most investigated theory. Here, both the extraneuronal and the intraneuronal environments can contribute to the breakdown of the ever important nigrostriatal pathway (4). Both environments contain the superoxide radical and nitric oxide (NO) and these are thought to interact both externally and internally (4) to produce peroxynitrite which can damage cellular components such as amino acids, proteins, enzymes, neurotransmitters, DNA, RNA and lipids (5). Consequently, of the various models used in PD research, the MPTP model is the model of choice to investigate the mechanism(s) involved in PD neurodegeneration (6). In humans, in non-human primates, and in other mammalian species, MPTP causes a severe parkinsonian syndrome that replicates almost all of the hallmarks of PD including tremor, rigidity, slowness of movement, postural instability and freezing. Both the responses to and the complications surrounding traditional PD medications are remarkably identical to those seen in PD. Mounting evidence such as the production of reactive oxygen species like the superoxide radical and reactive nitrogen species such as nitric oxide (NO) following MPTP administration support this oxidative stress hypothesis. Using transgenic mice that overexpress human copper/zinc superoxide dismutase (SOD1), the enzyme responsible for ridding the cell of the superoxide radical, we have demonstrated previously that the superoxide radical is indeed involved in the MPTP neurotoxic process (7). The superoxide radical has also been shown to be increased in various stroke models and in other neurodegenerative situations. In the past, Beckman et al (8, 9) have suggested that NO could be the other culprit involved in the oxidative stress hypothesis and that the superoxide radical and NO, only modestly reactive by themselves, but can react with each other to form the highly reactive, tissue damaging peroxynitrite. (8, 9). We have also found that mice engineered to express no neuronal NOS (nNOS) are only partially protected against the damaging effects of MPTP in the nigrostriatal DA pathway (10). Nitric oxide synthase (NOS) is the enzyme that produces NO. Three distinct isoforms of the NOS enzyme exist. Neuronal NOS (nNOS) is the principle NOS isoform in the brain and is constitively expressed throughout the

central nervous system (11) whereas endothelial NOS (eNOS) is found mainly in the endothelial layer of blood vessels and in very low concentrations in the brain (11). The third isoform of NOS, inducible NOS (iNOS), is not expressed at all or only minimally expressed in the brain (11). However, iNOS expression in the brain has been shown to be increased in pathological conditions such as stroke, AIDS and amyotrophic lateral sclerosis (12, 13, 14). In fact, iNOS expression has been demonstrated in the substantia nigra pars compacta (SNpc) of post-mortem brain tissues from PD patients (15) indicating that an inflammatory response may be part of the progressive nature of PD. From previous experience, since we and others have found that nNOS knockout mice are only partially protected against MPTP's toxic effects (10), we surmised that other NOS isoforms might indeed take part in MPTP-induced neurotoxicity. Therefore, based on the above information, it seemed germaine to our investigations into the causes of PD, to investigate the role of NO in the MPTP neurotoxic process.

STATEMENT OF WORK

Our overall long-term goal is the study of the pathogenesis of PD. To accomplish this, work in this project centers around the roles of the superoxide radical and, in particular, NO in the MPTP neurotoxic process. The basis of our work is the oxidative stress hypothesis of PD which supports free radical involvement in the etiology of and the progression of this debilitating disorder. Both the superoxide radical and NO are proposed as contributors to DA neuron death in the nigrostriatal DA pathway, however, each is only modestly reactive, but can combine to produce peroxynitrite, which damages proteins, DNA, polypeptides, enzymes and monoamines. Therefore, the overall objective of this project is to better understand the actual cascade of events that take place within the DA neuron following MPTP administration and which is ultimately responsible for the death of said neurons.

Research Plan

Specific Aim I: Determine the contribution of superoxide, NO or both to MPTP neurotoxicity by administering MPTP to different lines of mice which are genetically engineered to enhibit a greater capacity for detoxifying superoxide (transgenic copper/zinc superoxide dismutase {SOD1} mice) and/or a lower neuronal capacity for synthesizing NO (neuronal NO synthase {nNOS} knockout mice) and by assessing the status of the nigrostriatal DA pathway in these different types of animals following MPTP administration using high performance liquid chromatography (HPLC) and immunostaining with quantitative morphology.

Specific Aim II: Determine the contribution of inducible NOS (iNOS) to MPTP neurotoxicity by assessing iNOS protein expression and enzyme activity in different brain regions, at different time points and at different toxin concentrations in wild-type mice following MPTP administration. MPTP will also be administered to mice deficient in iNOS activity and the status of their nigrostriatal DA pathway will be assessed by HPLC and immunostaining with quantitative morphology.

Specific Aim III : Assess peroxynitrite effects on protein tyrosine residues following MPTP administration by quantifying the two main products of peroxynitrite exposure, that of dityrosine and nitrotyrosine by gas chromatography with mass spectrometry.

Quantification will be performed in different brain regions, at different time points and at different toxin concentrations in wild-type mice and in transgenic SOD1, iNOS and nNOS knockout mice.

Specific Aim IV: Examine the potential biological consequences of protein tyrosine nitration by assessing whether candidate proteins such as mitochondrial electron transport chain complexes and manganese superoxide dismutase (MnSOD) are nitrated. This will be tested in PC-12 cells after exposure to different concentrations of and lengths of time of peroxynitrate and MPTP's active metabolite, MPP+ as well as in wild-type and in transgenic SODI, iNOS and nNOS knockout mice after MPTP administration. Tyrosine nitration will be ascertained by immunoprecipitation and Western blot analyses. The catalytic activity of these enzymes in both PC-12 cells and in mice will be done using spectrophotometric enzymatic assays.

RESEARCH ACCOMPLISHMENTS

Year One of the Award (1999-2000).

During year I of the award, we addressed Specific Aim II which was to determine the source of NO in the SNpc following MPTP administration. Our major findings here were that **(1) MPTP produces a robust glial response**. To demonstrate this robust glial response, we used the macrophage antigen-1 (MAC-1) and glial fibrillary acidic protein (GFAP) as markers to gauge the responses of microglia and astrocytes, respectively, in the SNpc of C57/bl6 mice following MPTP administration. In saline-treated mice, only faint immunostaining for both MAC-1 and GFAP was observed. In the MPTP-treated animals, however, a strong glial response was observed. Alterations in MAC-1 were evident as early as 12 hours after the last dose of our acute MPTP regimen, peaked between 24 and 48 hours and was no different from saline-treated mice at 7 days after MPTP administration. Conversely, GFAP changes were noted at 24 hours, reached maximum increases between 4 and 7 days and remained above control levels even at 21 days after MPTP administration. Striatal responses for both antibodies were similar to those of the SNpc. We noted that **(2) MPTP stimulates iNOS expression in glial cells.** In saline-treated mice, whereas the number of iNOS-positive cells in the SNpc were rare to non-existent, iNOS-positive cell numbers increased to 259% of control by 24 hours after MPTP treatment and returned to control levels 48 hours later. Simultaneous staining techniques for iNOS and MAC-1 or GFAP were used to determine the nature of these iNOS-positive cells. Twenty-four hours after the last dose of MPTP at a time when the number of iNOS-positive cells reach their peak in the SNpc, MAC-1-positive activated microglia exhibited iNOS mmunoreactivity. No iNOS-positive staining was found in GFAP-positive cells nor were they found in the striatum. Also noted was the fact that **(3) iNOS mRNA levels and enzymatic activity increased dramatically after the acute regimen of MPTP administration.** Here, saline-treated ventral midbrain, which contains the SNpc, showed very little iNOS mRNA, whereas iNOS mRNA levels in ventral midbrain from MPTP-injected mice were detected as early as 12 hours ,reached maximum levels by 48 hours and was undetectable at 4 days after our acute regimen of MPTP administration. Striatal mRNA levels were low throughout the entire time course study. In agreement with mRNA levels, iNOS enzyme activity increases were evident as early as 12 hours after MPTP injections and peaked at 48 hours then slowly returned to

control activity by 7 days. iNOS striatal enzymatic activity like iNOS mRNA levels was unaffected by MPTP throughout the entire time course. In this study, nNOS enzyme activity was consistently higher than iNOS enzymatic activity as well as unchanged following MPTP administration. Since MPTP does increase iNOS expression and does up-regulate iNOS mRNA levels in normal wild-type mice after MPTP, absolute proof that iNOS is indeed the primary source of NO in the MPTP neurotoxic process was obtained using mice deficient in the iNOS enzyme. Administering the same regimen of MPTP to iNOS knockout mice and examining the same time points, we found that while 29% of the tyrosine hydroxylase (TH)-positive neurons and 46% of the Nissl-stained SNpc survived the toxic assault of MPTP in wild-type mice, about twice as many TH-positive and Nissl-stained neurons in iNOS deficient mice survived the MPTP onslaught indicating that **(4) iNOS is indeed the source of MPTP-induced NO production.** Interestingly, striatal fibres in the iNOS deficient mice exhibited the same level of loss as the MPTP-treated wild-type littermates. To make sure that the decreased loss of TH-positive neurons in the iNOS deficient mice was not related to alterations in MPTP uptake and metabolism due to the lack of the iNOS gene, we measured striatal MPTP and MPP+ levels in our iNOS knockout mice compared to their wild-type littermates. We observed **(5) no differences in MPTP and MPP+ levels in striata from these two groups of mice.** Finally, it is known that NO can damage DNA and nitrate the tyrosine residues in the phenolic rings of proteins and that nitrotyrosine (NT) is the indicator that NO has indeed reacted with the tyrosine residues of these proteins. In MPTP-treated wild-type littermates following MPTP, a significant presence of NT was noted in striatum and ventral midbrain. In contrast, in the iNOS deficient mice, whereas NT did increase, these increases were significantly less than those observed in the wild-type littermates. Thus, **(6) iNOS deficient mice have much less NT following our acute regimen of MPTP administration.** This entire work was published in the journal Nature Medicine in 1999 (Volume 5 (12), pp. 1403-1409.

During the first year of this award, we also addressed the question put forth in Specific Aim III, that of assessing peroxynitrite effects on protein tyrosine residues following MPTP administration by quantifying the two main products of peroxynitrite oxidation of tyrosine, dityrosine and nitrotyrosine using gas chromatography with mass spectrometry. Analyses of oxidized amino acids were performed on freshly isolated tissues from ventral midbrain which contains the SNpc, striatum, cerebellum and frontal cortex from and the compounds of interest had retention times that were identical to authentic 3-nitrotyrosine (3-NT), ortho-tyrosine and o,o-dityrosine. In these studies we found that **(1) 3-NT was elevated in ventral midbrain and striatum of mice as early as 24 hours after MPTP treatment.** Levels of 3-NT in ventral midbrain (+110%) and striatum (+90%) were markedly elevated following MPTP administration compared to saline-injected controls. It was noted that the observed increases were selective for regions of the brain that are susceptible to the neurotoxic effects of MPTP. Those regions that were not damaged by MPTP, cerebellum and frontal cortex, showed no changes in 3-NT levels. That **(2) o,o-dityrosine was elevated in ventral midbrain (+120%) and striatum (+170%) 24 hours after our acute regimen of MPTP administration was also noted.** These results were strikingly similar to the increases found in 3-NT and were found only in those regions of the brain that are affected by a toxic insult from MPTP. In contrast to the

changes in 3-NT and o,o-dityrosine found in ventral midbrain and striatum of these MPTP-treated mice, **(3) no changes in ortho-tyrosine were observed in any of the analyzed brain regions.** Since our theory is that the observed altered proteins were the result of exposure to peroxynitrite, we exposed, *in vitro*, homogenates prepared from ventral midbrain, striatum, cerebellum and frontal cortex to peroxynitrite, tyrosyl radical and the hydroxyl radical ($HO^{\cdot}$). **(4) Peroxynitrite exposure of the brain proteins for 30 minuites caused both significant and similar increases in 3-NT (80 fold) in all brain regions** indicating that peroxynitrite does indeed damage brain proteins. Ortho-tyrosine and o,o-dityrosine levels, however, increased only 2-3 fold. To generate the tyrosyl radical, we used an *in vitro* myeloperoxidase-tyrosine-H_2O_2 generation system, then exposed the various brain region homogenates to the generated tyrosyl radical. **(5) The major product of this reaction was o,o-dityrosine and all homogenates from the different brain regions exhibited similar increases in this compound. There were no changes in 3-NT or in ortho-tyrosine in any region.** Recent evidence suggests that the myeloperoxidase-H_2O_2 system will convert tyrosine into 3-NT in a reaction that requires nitrite, a degradation product of NO. Addition of nitrite to this system caused the appearance of 3-NT levels that were similar to those 3-NT levels found in mice following MPTP administration. To complete our studies here, we exposed our brain region homogenates to a $HO^{\cdot}$-generating system that contained copper and H_2O_2. In this system, **(5) while levels of 3-NT remained unchanged, levels of ortho-tyrosine and o,o-dityrosine increased significantly with ortho-tyrosine levels being 10-fold higher than those of o,o-dityrosine.** All three compounds can be considered markers of proteins damaged by exposure to NO in its various forms. This study was published in 1999 in the Journal of Biological Chemistry, Volume 274, pp. 34621-34628.

During year one of the Award, we investigated some of the proteins that were proported to play a role in PD. Alpha-synuclein was one such protein. The first clue linking alpha-synuclein to PD came through the discovery that point mutations in the alpha-synuclein gene caused an autosomal dominant Parkinson syndrome that is indistinguishable from sporadic PD (16, 17). The two most prominent mutations are the A53T (alanine to threonine change at the 53rd position) and the A30P (alanine to proline alteration at position 30) in the alpha-synuclein gene. Although the search for these mutation in sporadic PD failed, alpha synuclein has been identified as a major component of Lewy bodies which is considered a hallmark of PD. Furthermore, oxidative stress has been tied to damaged alpha-synuclein in the oxidative stress hypothesis of PD. Thus, we thought it possible that alpha-synuclein may participate in the degeneration of DA neurons in the SNpc. To address this question, we examined what happens to alpha-synuclein in the MPTP mouse model of PD. We found that following chronic MPTP administration, **(1) alpha-synuclein protein expression is increased in the ventral midbrain of the treated mice**. Expression steadily increased peaking at 4 days after the last dose of MPTP and then returned to control values. **(2) Increased expression paralleled the up-regulation of alpha-synuclein mRNA**. We also noted that **(3) this increase in expression was specific to the entire SNpc and predominant in the SN pars reticulata (SNpr).** In this study, we defined the temporal and topographical relationship between alpha-synuclein expression and SNpc DA neurodegeneration following MPTP administration. We did not see any changes in alpha-synuclein following our acute

regimen of MPTP; but only after our chronic regimen with this neurotoxin did we see changes in alpha-synuclein. This is an important finding because necrosis is the form of DA neuronal cell death following the acute regimen (18) whereas apoptosis is the dominant form of DA neuron death produced by the chronic regimen of MPTP administration (19). This finding speaks to the fact that it is the apoptotic form of DA neuron death that is thought to be the case in PD (20). The work in this study was published in the Journal of Neurochemistry, volume 74, pp 721-729 in 2000.

Year Two of the Award (2000-2001)

During year II of this award, since endothelial NOS (eNOS) is an isoform of the NOS enzyme, as part of Specific Aim I, we assessed the contribution of this isoform of NOS to the production of NO in the MPTP neurotoxic process. Our reasoning was that since the single main determining factor of the MPTP neurotoxic process is its conversion to MPP+ in glial cells in the brain, the absence of the eNOS gene could affect striatal blood flow thus compromising striatal MPP+ levels and the MPTP neurotoxic insult itself. For these studies, eNOS deficient mice and C57/bl mice from Jackson Laboratories (background of the eNOS-deficient mice) were given our acute regimen of MPTP (18mg/kg, i. p.) or saline at 2 h intervals and sacrificed at selected time points after the last MPTP injection. Brains were quickly removed and striatum, ventral midbrain, frontal cortex and cerebellum were dissected out, frozen on dry ice and stored at -80°C for Western blot analyses and monoamine and striatal MPP+ levels. Mice treated similarly were also sacrificed for fresh-frozen and perfused brains. **(1) Analysis of striata from eNOS deficient mice and their wild-type littermates revealed no significant differences in MPP+ levels between the two groups of mice**. Examination of ventral midbrain tissue optical density for Western blot analyses from eNOS deficient control, saline and MPTP-treated mice **(2) showed a specific band at 135 kDa indicating eNOS expression and that at no time during the time course study (0, 1, 2, 4, 7 days after MPTP administration) did MPTP alter eNOS protein expression levels.** Total mRNA was extracted from ventral midbrain from saline and MPTP-injected mice at the same time points as before for RT-PCR amplification and quantification of eNOS and GAPDH. Following amplification, electrophoresis, and exposure to radioactivity, quantification by optical density **(3) showed that the expression of eNOS mRNA remained unchanged throughout the entire MPTP time course.** To determine whether any change in eNOS immunostaining occurred in the SNPc, we performed immunohistochemistry for eNOS. **(4) In saline-injected mice, there was a dense network of eNOS positive blood vessels. Positive immunostaining showed a homogeneous distribution of blood vessels of varying sizes over the entire midbrain. No alternation in eNOS intensity of staining was seen in the midbrain after MPTP intoxication. In striatal sections, eNOS immunostaining was not as intense as in the midbrain. Also, no differences in eNOS immunoreactivity were noted among saline-injected and MPTP-treated mice at any timepoint. Results for NADPH histochemistry were similar to eNOS immunohistochemistry.** In any experiment involving MPTP, it is necessary to count the total number of TH-positive neurons that remain following the MPTP toxic insult. In this case, the total numbers of TH-positive and Nissl-stained neurons in the SNpc were counted using stereology. TH-and Nissl-stained neurons were counted in the right SNpc of every fourth section throughout the

entire extent of the SNpc. **(5) In wild-type and eNOS deficient mice, there was a large number of TH- positive cell bodies intermingled with a dense network of TH-positive fibers within the SNpc and there was no significant difference in the number of neurons between the two groups of saline-injected controls. In wild-type mice, 37 % of the SNpc neurons and 62% of the Nissl-stained SNpc neurons survived at 7 days after the 18 mg/kg acute MPTP regimen; the loss of both TH and Nissl-stained neurons in the eNOS deficient group was not statistically different from the wild-type group following MPTP dministration.** Thus, our conclusion from these experiments was that while nNOS and iNOS both play a role in the MPTP neurotoxic process, eNOS has no such role. Since this work produced a negative result, it was not published. However, attached are the figures demonstrating the non-involvement of eNOS in the demise of DA neurons in the nigrostriatal DA pathway of MPTP treated eNOS deficient mice and their wild-type littermates.

Since we have demonstrated that nNOS and iNOS are both involved in the MPTP neurotoxic process, that we can partially block the NOS enzyme with 7-nitroindazole, and that iNOS is the principal here, a logical extension of Specific Aim II would be the pharmacological blockade of iNOS upregulation. For this, we used minocycline a second-generation semi-synthetic tetracycline antibiotic, that is a potent inhibitor of microglial activation independent of any anti-microbial action. Its effectiveness as a neuroprotective agent was demonstrated against experimental brain ischaemia (21) and disease progression in the R6/2 mouse model of Huntington's disease (22). In the MPTP mouse model pf PD, minocycline **(1) attenuated MPTP-induced SNpc dopaminergic neurodegeneration.** Varying doses of minocycline 1.4-45 mg/kg x 2 daily) effectively increased significantly the number of surviving TH-positive neurons in the SNpc of mice given our acute regimen of MPTP (18 mg/kg x 4 doses over 8 hours). This protection was dose dependent in that while neuroprotection was not seen with 1.4 mg x 2 daily dosing, slight neuroprotection was seen with 5.625 mg/kg x 2 daily dosing and maximal protection (50%) occurred at 11.25 mg/kg x 2 daily and higher. Sparing of SNpc dopaminergic neurons does not always correlate with the sparing of their corresponding fibres which are essential for maintaining dopaminergic neurotransmission. Thus, we examined the striatal fibres for a neuroprotective effect using both the 18mg/kg and the 16 mg/kg acute dosing regimen dosage of MPTP and the varying doses of minocycline. We found **(2)that whereas minocycline offered striatal fibres no protection against the higher dose of MPTP, it did protect these fibres against the 16 mg/kg dose of MPTP.** A significant part of the MPTP neurotoxic process is mediated by NO-related oxidative damage the extent of which can be evaluated by assessing nitrityrosine formation. As before, we found that nitrotyrosine levels were significantly increased in ventral midbrain following MPTP administration. We also noted that these increases in nitrotyrosine levels were significantly smaller when minocycline in the presence of minocycline. Thus, **(3) in the presence of minocycline, MPTP-induced nitrotyrosine level increases were significantly smaller than in the non-minocycline-treated mice.** One can not establish with certainty that a compound is neuroprotective unless one demonstrates that the metabolism of the offending agent is not interfered with at any time along its metabolic pathway. In comparing striata from MPTP only with MPTP-minocycline treated mice, **(4) 90 mins after MPTP administration, results showed**

that striatal levels of MPP+ were not different between these two groups of mice. In experimental brain ischaemia, the neuroprotective effect of minocycline is reported to be due to its inhibition of microglial activation and proliferation. Part of the MPTP neurotoxic insult is a microglial response. Thus, we examined whether the neuroprotection afforded to SNpc dopaminergic neurons in the MPTP mouse model by minocycline is due to its inhibition of microglia activation. Mice were treated as per usual with the 18 mg/kg acute regimen of MPTP only or in combination with minocycline (45 mg/kg x 2 daily). As in previous experiments, MPTP alone elicited a robust microglial activation and a significant GFAP upregulation in the ventral midbrain 24 hours after the last injection. In mice treated with the minocycline-MPTP combination, **(5) although GFAP mRNA and immunostaining in the ventral midbrain and striatum remained as high and as intense as the MPTP only group, ventral midbrain MAC-1 immunostaining was similar to saline injected control mice.** Since minocycline attenuated the MPTP-induced microglial activation, we theorized that it should attenuate the production of some of the noxious mediators known to result from microglial activation. We found **(6) that the pro-inflammatory cytokine interleukin-1ßeta (IL-1ß) was indeed increased significantly following MPTP administration and that minocycline (45mg/kg x 2 daily) sigmificantly reduced this increase. Furthermore, the MPTP-induced upregulation of iNOS and NADPH oxidase, two prominent enzymes found in activated microglia that produce NO and reactive oxygen species (ROS), was completely abolished in the presence of minocycline.** This work was published in the Journal of Neuroscience, March1, 2002, pp 1763-1771.

Year Three of the Award (2001-2002)

The studies in wild-type C57/bl mice have essentially been completed. Since we have demonstrated that both the superoxide radical and NO are involved in the MPTP neurotoxic process, we next wished to dampen the effects of the MPTP toxic insult. We proposed to perform these studies using transgenic mice overexpressing wild-type SOD1, nNOS and iNOS knockout (ko) mice, and crosses between SOD1 and nNOS.ko and SOD1 and iNOS.ko mice. Because the first part of our studies were done using wild-type C57/bl mice, we transferred the SOD1 transgene into a C57/bl genetic background by applying the backcross system between hemizygote transgenic SOD1 mice and wild-type C57/bl mice at least seven times to assure that almost all of the alleles from the original strain were replaced followed by brother-sister matings. This breeding system took a little over one year and these animals are viable. Roughly 50% of each litter overexpress the SOD1 human transgene.

The goals of Specific Aim IV were to examine the biological consequences of protein nitration by assessing whether candidate proteins such as tyrosine hydroxylase, alpha-synuclein, MnSOD and mitochondrial electron chain polypeptides as well as any other proteins might be nitrated following MPTP intoxication. NO and ROS are both products of MPTP intoxication (7, 10, 23) and can react with each other to produce peroxynitrite which is known to damage proteins, DNA amono acids and even monoamines. We had noted in an earlier publication that alpha-synuclein is up-regulated in the entire SN and this up-regulation seemed to predominate in the cytoplasm of DA neurons (24). Using cell culture involving HEK293 cells transfected to overexpress the human alpha-

synuclein presynaptic protein, we found that **(1) following exposure to peroxynitrite, the alpha-synuclein in these cells was nitrated as demonstrated by Western blotting and immunoprecipitation techniques.** We surmised that the nitration of alpha-synuclein is likely due to the unstructured conformation of the protein in an aqueous solution. This exposes all four tyrosine residues in the alpha-synuclein to the solvent phase and increases the probability of the exposed tyrosines reacting with peroxynitrite. We replicated our in vitro findings in vivo in the MPTP mouse model as we saw **(2) nitration of alpha synuclein in MPTP-treated mice as early as 4 hours after the last dose of MPTP. No nitration of other presynaptic proteins such as ß-synuclein and synaptophysin was noted.** The nitration of alpha-synuclein is unclear however, it may have something to do with Lewy body formation and aggregation of proteins in the cell. This work was published in the Journal of Neurochemistry, volume 76, pp 637-640, 2001.

Since we noted that alpha-synuclein was nitrated in HEK293 cells following exposure to peroxynitrite and in ventral midbrain of mice treated with MPTP, we thought to examine whether tyrosine hydroxylase (TH) might be nitrated as early on we noted that tyrosine hydroxylase is down-regulated following MPTP. This is important because TH is the rate-limiting enzyme in catecholamine production. As with alpha-synuclein, **(1) exposure of TH to different concentrations of peroxynitrite produced a dose-dependent increase in the nitration of TH.** Furthermore, it was shown that **(2) tyrosine residue 423 was not only nitrated but was also the reason for the inactivation of TH following MPTP administration**. The nitration of TH and alpha-synuclein, proteins necessary for the normal operation of the nigrostriatal DA system, makes it apparent that abnormal nitration and oxidation reactions are widespread events in ventral midbrain of PD brains and in the MPTP mouse model of PD and may play key roles in the pathogenesis of PD. This work was published in the Journal of Biological Chemistry, volume 276, pp 46017-46023, 2001.

Although oxidation and nitration are processes germaine to the pathogenesis of PD in that these reactions alter several proteins necessary for the normal operation of the DA neuron, they are probably not the only events that are involved in the death of DA neurons. Mounting evidence (25, 26) indicate that a number of proteins from several different pathways may be in operation in the cell death process. Bax, a member of the Bcl_2 family of proteins, has emerged as a pro-cell death protein that may modulate or activate certain effectors of the cell death process such as caspases (27). It is known that Bax is required for the death of certain types of neurons in several disease states (28, 29, 30) including PD (20). Thus, we investigated the role of Bax in MPTP-induced DA neuron death. We found that as per double-labeling studies, **(1) the Bax protein is highly expressed in SNpc DA neurons.** Developmental studies show that **(2) Bax modulation of developmental cell death in the SNpc is dose-dependent** as Bax heterozygotes exhibit less cell death than Bax wild-types. Furthermore, Bax null mice show less cell death than Bax heterozygotes. In the studies with MPTP, we noted a **(3) dramatic up-regulation of both Bax protein and mRNA** and that **(4) Bax up-regulation paralleled the time course of MPTP-induced DA neuron death in the SNpc of the treated mice.** Bax modulation is related the presence of the Bax-Bcl_2 heterodimer (31). In this, Bcl_2

normally binds to Bax thus keeping Bax inactive. However, in the MPTP mouse model, **(5) MPTP decreases the dimerization of Bax with Bcl_2** as co-immunoprecipation studies showed a change in the ratio of Bcl_2 to Bax favoring much less Bax in the precipatate of the treated mice than in the control mce. In keeping with this, we showed that **(6) Bax null mice are resistant to the effects of MPTP in the SNpc.** These findings were made using the chronic MPTP mouse model which is a model of apoptotic neuron death in the SNpc. It is apoptosis and not necrosis that has been found to be the dominant form of cell death in PD (20). This work was published in Proceedings of the National Academy of Science, volume 98, pp 2837-2842.

Year Four of the Award (2002-2003).

In previous work, we determined the sources of NO to be both microglial (iNOS) and neuronal (nNOS). We next needed to find the source(s) of the superoxide radical as this radical could be a therapeutic target. In our earlier works, we showed that an inflammatory response in the SNpc following MPTP treatment was part of the neurotoxic process and we noted that the activated microglia here seemed to cluster around neurons. Thus, we thought to take a closer look at this inflammatory response. Evidence shows that NADPH oxidase is a significant source of ROS during inflammation (32). This multimeric enzyme is composed of 4 subunits (GP^{91phox}, p_{22phox}, p^{47phox} and p^{40phox}) and is inactive in resting microglia because p^{47phox}, p^{67phox} and p^{40phox} are all present in the cytosol as a complex and are separated from the transmembrane proteins, GP^{91phox} and p^{22phox}. When microglia become activated, p^{47phox} is phosphorylated and the entire complex translocates to the plasma membrane where it assembles with GP^{91phox} and p^{22phox} to form the NADPH oxidase complex. which reduces oxygen to the superoxide radical. This radical can, in turn, give rise to other ROS (32). Thus, we examined NADPH oxidase in the face of acute MPTP administration. We found that **(1) NADPH oxidase as measured by GP^{91} immunoreactivity is induced in the ventral midbrain of MPTP-treated mice.** This enzyme was shown to peak at 24-48 hours after MPTP which is in keeping with the time course of microglial activation. Furthermore, we show that **(2) NADPH oxidase is expressed in activated microglia following MPTP administration in both the striatum and the SNpc and seems to co-localize with MAC-1 immunostaining.** It does not, however, co-localize with glial fibrillary acidic protein (GFAP), a marker for astrocytic activation. On ethidium fluorescence, we **(3) demonstrated a significant presence of the superoxide radical in the SNpc of wild-type mice whereas in the SNpc of GP^{91}knockout mice shows significantly less superoxide production following MPTP treatment.** We also noted that **(4) the SNpc in GP^{91}knockout mice is protected against the damaging effects of MPTP** as this structure showed significantly more surviving TH-positive neurons. Moreover, we found that **(5) the GP^{91} subunit of NADPH oxidase is increased in PD brains**. This evidence weighs heavily in favor of the oxidative stress hypothesis of PD and was published in the Proceedings of the National Academy of Science, volume100, pp 6145-6150, 2003.

In keeping with our premise of the oxidative stress hypothesis of PD, we noted that epidemiological studies suggest that inflammation can increase the risk of developing a neurodegenerative disorder such as Alzheimer's disease. In following this line of thought, inflammatory processes associated with increased expression of cyclooxygenase-2

(COX-2) and elevated levels of prostaglandin E_2 (PGE_2) are thought to be part of a series of events that lead to neurodegeneration in several disease states (33; 34). COX-2 and PGE_2 are both found in DA neurons. DA is a relatively unstable molecule and can be 1) subjected to hydroxyl radical attack (35) and nitrated within its own neuron (36), as well as oxidized to DA-o-quinone which, upon addition of a sulfhydryl group from cysteine, becomes 5-cysteinyl-DA (37). We recently investigated the relationship between 5-cysteinyl-DA and the neurodegeneration seen in PD in the MPTP mouse model of PD. In our experiments, we show that **(1) MPTP induces COX-2 expression and activity in the ventral midbrain of the treated mice.** Barely detectable in saline-treated mice, COX-2 was detected in the SNpc 24 hours after injections and was still present 7 days later. **(2) Ventral midbrain PGE_2 levels were shown to increase in a fashion similar to that of COX-2 following MPTP administration and it was determined that COX-2 was the primary source of the increases seen in PGE_2.** We also saw **(3) COX-2 positive staining in several neurons in the SNpc after MPTP administration and co-localization studies showed that these neurons were also TH-positive.** Furthermore, we show that. **(4) COX-2 is up-regulated in postmortem tissues from PD brains.** The definitive evidence that COX-2 is indeed involved in SNpc DA neuron death was substantiated in mice deficient in the Cox-2 enzyme. In these mice, following MPTP administration, **(5) the SNpc showed a significant number of surviving TH-positive neurons(63%) when compared to heterozygotes (21%) and wild-type mice (16%).** Moreover, since stress can activate Jun kinase (JNK) in mammalian cells (38), we examined the COX-2 enzyme in MPTP-treated COX-2 deficient mice in which the COX-2 enzyme was blocked by CEP-11004, a JNK inhibitor. Here, we noted that **(6) although the SNpc was protected against MPTP in CEP-11004-treated COX-2 deficient mice, there was no difference between CEP-11004-treated and untreated mice COX-2 deficient mice.** The last piece of the puzzle was the demonstration of the presence of 5-cysteinyl-DA following MPTP administration. Using HPLC, we **(7) demonstrated a twofold increase in the presence of 5-cysteinyl-DA, an oxidized form of DA, in the striatum.** Thus, this study shows that the both the COX-2 and PGE2 enzymes are a part of the MPTP neurotoxic process and the PD disease process. This work was published in the Proceedings of the National Academy of Science, volume 100, pp 5473-5478.

In DA neurons, at the cellular level, mitochondria play a huge role in the stability of intracellular environment. Part of the oxidative stress hypothesis of the death of DA neurons in the SNpc is that there may be a defect in oxidative phosphorylation. A reduction of complex I (NADH-ubiquinone oxidereductase) of the mitochondrial electron transport chain is one of the salient features of PD and defects in tissues other than brain have been found (39). Supporting the idea of a defect here is the fact that MPP+, the active metabolite of MPTP, blocks complex I as seen in PD (6). It is known that alternate energy sources like creatine can attenuate DA neuron death by improving oxidative phosphorylation (40). D-ß-hydroxybutyrate (DßHB) is a ketone body that is produced by hepatocytes and astrocytes and is an alternative source of energy in the brain when the glucose supply is depleted such as during starvation. DßHB has been shown to prevent damage to neurons that occurs following starvation (41) and mitochondrial poison exposure (42). Thus, we investigated this compound in our MPTP mouse model of PD. Mice were given 3-nitroproprionic acid (3-NP, to block complex II but not to cause cell

damage) 2 hours before implantation of Alzet minipumps containing varying doses of DßHB. Twenty-four hous later, mice were injected with our acute regimen of MPTP and sacrificed at varying timepoints after MPTP injections. In these experiments, we found that **(1) MPTP upregulates the DßHB metabolizing enzyme and increases the utilization of DßHB in the brain.** DßHB crosses the blood-brain barrier and enters mitochondria where it is metabolized by ß-hydroxybuterate dehydrogenase to acetoacetate. Acetoacetate is converted to acetyl-CoA which then enters the Krebs cycle. Following infusion, brain levels of DßHB were higher in saline-treated than in either MPTP or 3-NP-treated mice which is indicative of increased usage. We also noted that **(2) DßHB attenuates MPTP-induced DA neuron degeneration in the SNpc as well as DA terminal loss in the striatum** when compared to vehicle-treated mice. Furthermore, this compound not only protected against structural damage but also against functional damage in the nigrostriatal DA pathway as **(3) DA and its metabolite levels were significantly higher in DßHB-infused MPTP-treated mice than in the MPTP only animals**. Because of the protection afforded by DßHB here, **(4) this translated into a better performance of the rotarod for the DßHB-infused MPTP-treated mice.** When complex 1 is blocked as is the situation with MPTP, mitochondrial function is hampered and there is a significant decrease in oxygen utilization. Consistent with DßHB being a mitochondrial substrate, we found that **(5) DßHB restored completely oxygen utilization in the presence of MPP+, and this action was stereospecific as the L form of ßHB is ineffective on reversing the changes in oxygen consumption induced by MPP+**. We noted that this effect was dose-dependent. Because it could be surmised that this reversal effect might be due to the uncoupling of the mitochondria or an artifact, we examined mitochondrial membrane potential and found that **(6) even high concentrations of DßHB had no effect on mitochondrial membrane potential.** Finally, we needed to determine how DßHB rescued mitochondrial respiration. DßHB can generate succinate which is able to increase the rate of oxygen utilization in isolated mitochondria through complex II. Thus, **(7) in our 3-NP-treated MPTP injected mice, DßHB failed to protect DA neurons whereas these neurons were protected in MPTP-treated mice that received that were infused with DßHB.** Therefore, DßHB, an alternate source of glucose for brain energy, can protect the nigrostriatal DA pathway against the damaging effects of MPTP through a mechanism involving succinate and improved mitochondrial respiration.

As mentioned above, defects in oxidative phosphorylation and ATP production are a part of the PD picture and part of the MPTP-induced detrimental profile. Aside from alternate energy sources as corrective measures for these defects, overexpression of certain mitochondrial enzymes can attenuate mitochondrial defects. Type II L-3-hydroxyacyl-CoA dehydrogenase/amyloid ß peptide binding alcohol dehydrogenase (HADH II/ABAD) is predominantly a mitochondrial enzyme that belongs to the short-chain dehydrogenase/reductase superfamily (43). This enzyme converts L-3-hydroxyacyl-CoA to 3-ketoacyl-CoA, NADH and H+ in the presence of NAD+ and appears to be a multifunctional enzyme with a wide range of substrates (44). Overexpression of HADH II/ABAD has been shown to reduce infarct size and neurological deficits in cerebral ischemia by feeding into the Krebs cycle and by increasing ATP levels in the brain (45). Most the proctecive effects of HADH II/ABAD have been demonstrated in acute models,

but since PD is a slow progressive disorder, we reasoned that the overexpression of HADH II/ABAD might attenuate some of the MPTP-induced detrimental profile. Thus, mice that overexpressed human HADH II/ABAD were subjected to our chronic regimen of MPTP administration and sacrificed at various times during and after MPTP administration. Relevant to PD, on Western blot analysis, we found **(1) that HADH II/ABAD is severely reduced in the ventral midbrain from PD patients but is normal in striatal tissues from these same brains.** This finding was confirmed by immunohistochemical studies. Since the MPTP mouse model is a replica of PD, we examined HADH II/ABAD protein in MPTP-treated mice and found **(2) that ventral midbrain HADH II/ABAD protein in wild-type mice was indeed reduced following the chronic regimen of MPTP treatment although TH protein levels were not.** Maximum reduction occurred at 2 and 7 days after MPTP administration which corresponds to the active phase of SNpc DA neuron death in this model. In contrast to wild-type mice, **(3) HADH II/ABAD mice exhibited a higher optical density for TH-positive fibres and showed a significant increase in the number of surviving DA neurons; also noted were fewer apoptotic neurons in the SNpc.** In contrast to its structural effects in the nigrostriatal DA pathway, **(4) HADH II/ABAD did not protect here functionally as DA and its metabolites were not different from wild-type MPTP-treated animals.** In our investigations of mitochondria from HADH II/ABAD overexpressers and their wild-type littermates, we found **(5) that MPP+ inhibited mitochondrial oxygen consumption mediated by complex I in the wild-type littermates but was less effective in this action in HADH II/ABAD overexpressers.** Higher oxygen consumption in coupled mitochondria translates into higher ATP production and consistent with this, **(6) HADH II/ABAD mitochondria produced more ATP than wild-type littermate mitochondria,** thus, HADH II/ABAD is able to sustain mitochondrial respiration. As to how this is done led us to assess the intramitochondrial distribution of HADH II/ABAD. We found that **(7) in normal respiring mitochondria, the majority of HADH II/ABAD resides in the mitochondrial matrix and only small amounts are in the mitochondrial membrane. When incubated with MPP+ which inhibits complex I, HADH II/ABAD content in the mitochondrial membrane increases so that this enzyme can be in close proximity to complex I. This increase is the result of a translocation of HADH II/ABAD from the mitochondrial matrix to the mitochondrial membrane.** From these studies, we conclude that HADH II/ABAD acts as an alternate energy booster in conferring its neuroprotective effect on SNpc DA neurons. This work was published in Annals of Neurology, volume 56, pp 51-60 in 2004.

SHORTFALLS IN THIS AWARD.

The breeding of nNOS knockout mice was not as straightforward as we thought it would be. These mice were originally in a mixed 129 SvEv agouti -C57/bl background thus we first had to transfer the nNOS.ko gene into a C57/bl background using the backcross and brother-sister mating system. We bred the few homozygote nNOS.ko mice that we had with C57/bl females (Jackson Labs) to obtain nNOS heterozygotes. Brother-sister mating of the resulting heterozygotes was then done to obtain nNOS.ko mice. What we found was that this crossing of heterozygote x heterozygote did not respect Mendalian genetics (25/50/25). Although they were viable from our breeding program, we were lucky if one mouse per litter was a nNOS.ko mouse. Thus acquiring the numbers needed was not so

fruitful. After a number of failed attempts, we decided to try and buy these animals ($338.00 per breeding pair) from Jackson Labs. We spent a number of months going back and forth with them only to be informed that they were having problems with their colonies of nNOS.ko mice and that we would have to wait until they solved their problems with this colony. In April of 2003, Jackson Labs informed us that they had cryopreserved this line of mice. We assumed that this cryopreservation occurred because they did not solve their colony problems. We thus have looked to other investigators who may have these mice and who were maybe more successful in their breeding programs. This approach has been partially successful in that we have now been promised nNOS.ko mice. Recently, Jackson Laboratories have contacted us and said that they can supply us with nNOS.ko mice within four months. Our attempts to crossbred SOD1 with nNOS.ko was also a problem in that this $SOD1^{+}/nNOS^{-/-}$ cross was indeed a rarity per litter.

The breeding of iNOS ko mice proved to be almost as problematic as the nNOS.ko mice. Litter size was usually 8-10 pups however, mothers in many cases ate their young or did not care for them. Thus, a significant number of iNOS pups died before they reached weaning age. Jackson Labs carries this line of mice also, so we decided to purchase adult iNOS.ko mice from this vender. Production of these mice, already in a C57/bl background, at Jackson Labs is, however, intermittent and limited; one is now allowed to purchase only 10 iNOS.ko mice at any one time making it difficult to obtain sufficient numbers for any one experiment. Crosses with SOD1 were problematic as well yielding maybe 1 to none $SOD1^{+}/iNOS^{-/-}$ mouse per litter. While we were conducting the breeding program, and are still attempting to get around this problem of the genetically engineered mice, we published several reviews and editorials in peer-reviewed journals that are relevant to the subject of the role of NO in PD and MPTP neurotoxicity.

Extension Year of the Award (2004)

RECOVERY

The work in the genetically engineered mice is still yet to be completed. In fact, because of the breeding problems, we are forced to investigate other avenues, both mammalian and pharmacological, to make up for these shortfalls. While SOD1 overexpressers pose no great problem, nNOS and iNOS do. Breeding of these mice was far less than fruitful. We have, however, been promised a limited number of nNOS.ko mice and we can now purchase very limited numbers of iNOS.ko mice. However, this does not solve the total problem of completing **Specific Aim I**. Since we have examined the effects of a SOD mimetic in MPTP-treated mice in another set of studies and found that this compound can attenuate significantly the damaging effects of MPTP in the SNpc of mice, we feel that M40401 or similar compounds may be a viable solution to one aspect of the genetically engineered mouse problem, that of iNOS and nNOS and their crosses with SOD overexpressers. Thus, our original thought was to administer M40401 to iNOS and nNOS knockout mice. Although M40401 is a manganese SOD mimetic, it is the only SOD mimetic that we have used thus far that can cross the blood-brain barrier and that can attenuate the damaging effects of MPTP in the mouse SNpc (manuscript in preparation). We have infused CuZnSOD into the striatum of MPTP-treated mice (46) because it does

not cross the blood-brain barrier but it does confer neuroprotection. In our attempts to obtain M40401 to complete our studies, a problem has recently occurred between our supplier of this compound and the supplier's company which has interrupted our supply of M40401 thus hindering our planned studies with this compound. So, we are now looking for other SOD mimetics to administer to the iNOS and nNOS knockout mice. To approach the problem of blocking iNOS upregulation in SOD1 mice, in previous studies, we have used minocycline, a second generation tetracycline antibiotic, to block iNOS up-regulation in C57bl/6 MPTP-treated mice. Minocycline decreased microglial activation and attenuated MPTP-induced damage to the SNpc (47). So, minocycline seems to be a viable option in SOD1 MPTP-treated mice to get around the problem of the SOD1-iNOS.ko cross. Although a study following our study has demonstrated that minocycline exaserbates MPTP-induced DA neuron death in the nigrostriatal DA pathway (Beal et al-48), with our regimen, we found no such results. For the nNOS crosses in the SOD1 mice, in prior studies, we successfully blocked nNOS up-regulation using 7-nitroindazole, a compound that blocks the up-regulation of the nNOS enzyme (10). However, 7-NI also inhibits monoamie oxidase activity (49) which poses a problem for these experiments. Therefore, we are trying to obtain a more specific nNOS inhibitor to examine that part of our original question on the role of NO in MPTP-induced dopaminergic neuron degeneration. We will also administer minocycline and a new nNOS inhibitor to SOD1 overexpressers. But, before using any of the few transgenic or knockout mice that we may obtain, and in order for us to perform these studies, we needed to test the feasibility of using smaller-sized samples and to gauge levels of detectability in these smaller-sized samples. Thus, over the extension year (2004), we administered MPTP to normal C57bl/6 mice and split the brains evenly down the middle. One-half of the brain was immersed in 4% paraformaldehyde/0.1M phosphate buffer for various times to obtain optimal immunostaining conditions for TH as well as MAC-1 and GFAP immunostaining. We found that 16 hours of immersion in paraformaldehyde followed by 48 hours of cryoprotection is suitable for all three staining procedures in that we found no differences in results between the perfused tissues versus the immersed tissues. The other half of the brain was dissected (striatum and ventral midbrain) and tested for feasibility of use in catecholamine and Western blot analyses. This approach can also be done. Thus, we believe that we can perform the necessary studies for **Specific Aim I.** For **Specefic Aim III**, we will attempt to assess peroxynitrite effects on protein tyrosine residues in half of the amount of sample from iNOS and nNOS knockout mice following MPTP, in a few engineered mice treated with MPTP in combination with a SOD mimetic and in SOD1 overexpressers treated with minocycline or a specific nNOS inhibitor. We will also attempt to quantify the two main products of peroxynitrite oxidation of tyrosine, dityrosine and nitrotyrosine in different brain regions (striatum, ventral midbrain, frontal cortex, berebellum) using half samples. Because of the limited tissue availability, several timepoints may not be doable, but we will try for as many timepoints as we can. For **Specific Aim IV**, we will examine the consequences of protein tyrosine nitration by assessing whether candidate proteins, such as mitochondrial electron transport chain polypeptides and manganese-SOD are nitrated. This will be done using the other half-brain tissues from **Specific Aim III.** Tyrosine residue nitration in mitochondria will be determined by immunoprecipitation and Western blot analyses following use of the immunocapture procedure (Mitosciences) to concentrate said

proteins and Western blot analyses for quantification. We have spent a good part of last year searching for a method and learning the above-mentioned procedure. We will make all attempts to complete this project as we feel obligated to the US Army and to those who suffer from PD.

In SAIII, we found marked increases in O,O'-dityrosine content in the SNpc of MPTP-treated mice as well as elevated levels of 3-chlorotyrosine (3-CT) and 3-NT (50). Since O,O'-dityrosine is reported to be a relatively minor product of peroxynitrite, it was suggested that that the increases in these markers may be caused by some other oxidant-producing enzyme related to the inflammatory response. Myeloperoxidase (MPO) may be one such enzyme. It can use the NO degradation product NO_2 to generate RNS and prior studies using MPO deficient mice have shown that MPO is one of the major sources of 3-NT during acute inflammation (51). Furthermore, since MPO is a heme protein enzyme that is expressed in abundance in phagocytic cells, it is possible that as part of the inflammatory response, MPO could contribute to the degenerative process in the SNpc of MPTP-treated mice and in PD. Thus, the logical extension of our finding was to determine the role of MPO in SNpc DA neurodegeneration and the source of the dityrosine, 3-CT and 3-NT alterations. In experiments using our acute regimen of MPTP administration and our standard schedule of sacrifice (0, 12hr, 1, 2, 4 and 7 days after MPTP administration), we demonstrated **(1) that MPO is induced in ventral midbrain of MPTP-treated mice during the degeneration of SNpc DA neurons.** MPO mRNA and protein expression levels in the ventral midbrain of MPTP-treated mice peaked at 1-2 days after MPTP administration, the same timeframe as the most intense phase of DA neuron degeneration and the peak of the MPTP-induced inflammatory response. We noted **(2) that MPO is strongly expressed in reactive astrocytes at the level of the SNpc.** Immunofluorescence studies with GFAP, an astrocytic cell antibody, confirmed astrocytes as the site of production for MPO. We also investigated our premise about MPO in postmortem tissues from PD brains and found **(3) that MPO immunoreactivity is increased in the PD midbrain relative to control brains.** Since we found elevated MPO expression in the caudate nucleus from Huntington disease brains, we believe that these increases may be common to any neurodegenerative process accompanied by a glial response. Moreover, we documented **(4) that MPO damages ventral midbrain proteins** using the HOP-1 antibody. that specifically recognizes HOCL (hypochlorous acid) modified proteins. Thus, we conclude that MPO is responsible for the markers of the modified proteins, dityrosine, 3-CT and 3-NT and that these alterations occur because of secondary ROS and RNS production during the inflammatory process in the MPTP model and in PD. This work was published in the Journal of Neuroscience, volume 25, pp 6594-6600 in 2005.

KEY ACCOMPLISHMENTS RESULTING FROM THIS 4 YEAR AWARD.

Specific Aim I

Both the iNOS and nNOS enzymes are involved in the MPTP neurotoxic process. The SNpc of both iNOS and nNOS knockout mice is only partially protected against the damaging effects of MPTP.

eNOS is expressed in blood vessels in the brain, but is not involved in MPTP neurotoxic process in the SNpc as (1) content and peak time of MPP+ levels show no differences between wild-type and eNOS deficient mice; (2) mRNA and protein levels of the eNOS gene are unchanged in the ventral midbrain following MPTP treatment in eNOS deficient mice; (3) the loss of both TH.- and Nissl-stained neurons between wild-type and eNOS deficient mice were not statistically different following MPTP administration.

NADPH oxidase is induced in the SNpc of MPTP-treated mice, is expressed in activated microglia and is the source of the superoxide radical in MPTP-mediated microglial activation. Subunits of NADPH oxidase such as GP^{91} is also up-regulated in the SNpc in PD and following MPTP; inactivation of NADPH attenuates the MPTP effect by mitigating inflammation..

Devised methods using immersed half-brain for morphological studies and half-brain dissected tissues for biochemical and morphological studies that show no differences from our established methods. We will attempt to use these devised methods to complete the studies for Specific Aim I.

Specific Aim II

iNOS seems to be the main NOS enzyme and the main producer of the NO involved in the MPTP neurotoxicity and degeneration of DA neurons ; it is up-regulated in microglia in the Snpc of MPTP-treated mice. iNOS mRNA is also up-regulated here.

MPTP-induced toxicity in the SNpc can be attenuated with minocycline, a second generation semisynthetic tetracycline antibiotic. Minocycline inhibits the inflammatory response in the SNpc induced by MPTP. This effect is independent of its antimicrobial activity. Minocycline prevents three key microglial-derived mediators of cytotoxicity following MPTP administration: iNOS upregulation, formation of mature IL-1ß and activation of NADPH oxidase.

Cyclooxygenase 2 (COX-2) enzyme expression is induced within SNpc DA neurons in postmortem PD samples and in the SNpc of MPTP-treated mice during the degenerative process. This up-regulation of the COX-2 enzyme occurs through a c-jun kinase (JNK)/ c-jun dependent mechanism. Both ablation and inhibition of COX-2 attenuate MPTP-induced DA neuron degeneration in the SNpc of mice possibly by decreasing PGE2 up-regulation which prevents the oxidative modification of DA to 5-cysteinyl-DA.

Specific Aim III

Documentation of the existence of peroxynitrite and the regional quantification of protein oxidation markers 3-nitrotyrosine, o,o-dityrosine and orthotyrosine in the ventral midbrain from the MPTP mouse model of Parkinson's disease. This suggests both tyrosyl radical and peroxynitrite involvement in the neurodegenerative process induced by MPTP and that protein oxidation and

nitration seen in the MPTP model may be mediated through the myeloperoxidase (MPO) heme protein secreted by activated phagocytes.

Documentation and demonstration that myeloperoxidase (MPO), the above-mentioned heme protein is up-regulated in the mouse ventral midbrain during MPTP-induced DA neuron degeneration. Increased expression of this enzyme is also found in PD brains. MPO is the only known source of hypochlorous acid (HOCl) that reacts with tyrosine to form 3-chlorotyrosine, a specific stable marker of protein damaged by MPO. Using the HOP-1 antibody which recognizes HOCL-modified proteins, we showed HOP-1 positive staining in the SNpc of MPTP-treated mice. Demonstration that ablation of MPO protects DA neurons in the SNpc against MPTP-induced degeneration.

Devised methods using immersed half-brain for immunostaining and half-brain tissues for biochemical and morphological studies that show no differences from our established methods. We will attempt to use these devised methods to complete the studies for Specific Aim III.

Specific Aim IV

Examination and demonstration of the inactivation of tyrosine hydroxylase as a result of the nitration of tyrosine residues in HEK293 cells exposed to various concentrations of peroxynitrite and in ventral midbrain from MPTP-treated mice.

Demonstrated the up-regulation, nitration and oxidative modification of alpha synuclein protein in neurons in the SNpc of MPTP-treated mice. All synuclein-positive neurons were also TH-positive

Showed that the blockade of the complex I enzyme by MPTP, which increases the production of the superoxide radical, can be overcome by infusion of the ketone body D-ß-hydroxybutyrate (D-ßHB) which enhances the functional operation of complex II thus partially protecting against MPTP-induced DA neuron degeneration, by improving mitochondrial respiration and increasing ATP production. This suggests that the complex I enzyme, a possible candidate protein for interaction with NO, may be nitrated thus prevented from passing electrons down the mitochondrial chain.

Demonstration that defects in mitochondrial respiration can also be overcomed by the overexpression of HADH II/ABAD, a mitochondrial matrix enzyme that can translocates to the mitochondrial membrane thus attenuating the defects in mitochondrial respiration caused by MPTP.

Worked out methods (immunocapture with Western blot analyses) for identifying mitochondrial candidate proteins (complex I and manganese SOD) that may be nitrated following MPTP administration. We will use these methods in attempts to complete the studies for Specific Aim IV.

REPORTED OUTCOMES FROM THIS 4 YEAR AWARD.

Manuscripts

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CONCLUSION

Parkinson's disease is a common neurodegenerative disorder that affects a reasonable percentage of our aging population (1). Possessing a tool like MPTP that can replicate almost all of the hallmarks of this disorder has indeed been a gift. Finding the cause of this debilitating disease and elucidating the molecular mechanisms that are involved in the death of dopaminergic (DA) neurons in the SNpc can give us specific targets for therapeutic strategies aimed at abating DA cell loss and the results of this loss. In keeping with our original hypothesis of free radical participation in the death of these specific neurons (52), using the MPTP mouse model of PD, we have found that both the superoxide radical and NO are indeed involved in the death of DA neurons in the SNpc of MPTP-treated mice. Sources of the superoxide radical are several, mitochondria, NADPH oxidase and PGE_2, and are both intracellular and extracellular. In elucidating the role of NO in the MPTP-neurotoxic process, it was necessary to sort out, first of all, which of the three nitric oxide synthase (NOS) isoenzymes actually contribute to the MPTP neurotoxic process in DA neurons in the SNpc. Our studies indicate that of the three, nNOS (NOS1) and iNOS (NOS2) are involved here (10, 23), but eNOS (NOS3) is not (Wu and Przedborski, unpublished data). Given this, it is reasonable to think that DA neurons are more susceptible to oxidative stress because of the simultaneous perturbations in their internal and external environments caused by superoxide and nitric oxide.

The question is, what is the environment in and around the DA neuron in the SNpc and just how does its environment play a significant role in its own demise. Although both nNOS and iNOS are up-regulated, we have demonstrated that the NOS2 enzyme in the SNpc seems to be the main NOS culprit in PD and in the MPTP mouse model and this culprit comes with heavy baggage. First of all, as stated above, MPTP induces an increased expression of the NOS2 enxyme in the SNpc of the treated mice; up-regulation of NOS 2 has also been found in postmortem tissue samples from PD brains (23). Increased expression of NOS2 and increases in the production of the super- oxide radical

are the result of the activation of microglia (23, 47) which is a response to neuronal injury. And, it is the neuronal injury that provokes and keeps the inflammatory process afloat which makes this situation a circular process. Coupled with the up-regulation of NOSII in microglia following MPTP administration, we observed increased expression of NADPH oxidase in microglia (46). Immunofluorescence studies have shown that NADPH oxidase promotes the production of the superoxide radical which was shown to be present in microglia using hydroethidium fluorescence (46). Thus, the microglia are the forum for a superoxide radical/NO clash in the MPTP mouse model and possibly in PD itself. The interaction of these two "radicals" most likely results in the formation of peroxynitrite, a compound that can modify, thus inactivate amino acids, proteins and catecholamines (8, 9) whose physical presence cannot be demonstrated. But, microglia are not the only cells in the SNpc that produce the superoxide radical and NO. Neurons themselves are also involved in their own demise. From our studies (53) as well as from studies by other investigators (54, 55, 56), it was shown that the MPTP metabolite, MPP^+, blocks mitochondrial complex I of the mitochondrial electron transport chain within the neuron which also kicks out the superoxide radical. Since NO can travel as many as 300 microns from its site of production and can freely travel through membranes (57), we conclude that the superoxide radical and NO can also interact within the neuron to produce peroxynitrite thus causing its own demise. Peroxynitrite can therefore be formed both inside of and outside of the DA neuron. Inhibition of either the superoxide radical (transgenic SOD1 overexpression) or NO formation (minocycline, 7-nitroindazole) can diminish the damage caused by the interaction of these two compounds in the MPTP mouse model.

A quick, cheap and easy way to end-run the MPP^+ inactivation/block of complex I as well as to overcome the reduction in complex 1 activity seen in PD is to circumvent the block and the reduction of complex 1 possibly through the application of the ketone body, D-ß-hydroxybutyrate (D-ßHB) (58). D-ßHB is producesd in the hepatocytes and, to a lesser extent, in astrocytes (ref). It can act as an alternative source of energy in the brain when the glucose supply is depleted such as during starvation. In vitro, D-ßHB prevents neuronal damage following glucose deprivation and mitochondrial poison exposure. In our MPTP mouse model, we have documented that the infusion of D-ßHB protects SNpc DA neurons in a dose-dependent and stereo-specific manner and prevents the development of PD-like motor abnormalities. D-ßHB seems to enhance oxidative phosphorylation by a mechanism dependent on succinate ubiquinone oxidoreductase (complex II).

As stated above, SNpc neurons in PD and in the MPTP mouse may contribute to their own destruction. The superoxide/NO interaction is not the only source of inflammation within the DA neuron. The COX-2 enzyme is thought to play a role in inflammation within the DA neuron as well, for as we have shown, up-regulation of the COX-2 enzyme within the neuron is thought to be one event among a cascade of deleterious events in the neurodegenerative process (59). In the inflammatory process within the DA neuron, it had been suggested that the increases in the expression of the COX-2 enzyme is responsible for the elevated levels of prostaglandins particularly prostaglandin E2 which has been associated with neurodegeneration. In our studies using the MPTP mouse model

of PD and SNpc tissue samples from PD brains, we found induction of the COX-2 enzyme and elevation of its catalytic activity in DA neurons of the SNpc of MPTP-treated mice and in the PD tissues. We also noted that this induction was absent in MPTP treated COX-2 knockout mice, in MPTP mice treated with Refocoxib, a specific COX-2 inhibitor as well as in mice treated with the JNK inhibitor CEP11004 (59). What was interesting in these experiments was that neither ablation nor inhibition of the COX-2 enzyme affected the activation of microglia by MPTP in the SNpc (23). From these experiments, we concluded that the JNK/c-jun signaling pathway is instrumental in COX-2 enzyme induction within the DA neuron, that induction of this enzyme is responsible for elevated PGE2 levels, that this sequence of events has a role in the inflammatory process inside of the DA neuron and that these events do not involve activated microglia.

Peroxynitrite is a very short-lived elusive compound that, in reality, cannot be physically measured. It is formed intracellularly within mitochondria and, can diffuse in and out of mitochondria and can undergo targeted molecule reactions with various cellular compounds such as proteins, amino acids and poly peptides as well as react with carbon dioxide to yield secondary radicals which participate in the oxidation, nitration or nitrosation of critical mitochondrial components. Specific markers of peroxynitrite interaction attest to the damage it can inflict. In keeping with our original hypothesis about the role of NO in PD and in the MPTP neurotoxic process, and after having shown that during the MPTP neurotoxic process that TH was nitrated (60), we also demonstrated *in vitro* that peroxynitrite can nitrate tyrosine residues in the TH molecule and concluded from these studies that tyrosine 423 was the primary tyrosine that was nitrated. Nitration here is sufficient to inactivate the TH enzyme (61). We also noted that other molecules are nitrated by peroxynitrite. For instance, the synaptic protein, alpha synuclein, whose role in the degeneration of DA neurons has yet to be elucidated, is not only up-regulated in the SNpc of MPTP-treated mice (24), but also is nitrated and oxidatively modified following the administration of this toxin (62). Based on the fact that peroxynitrite has access to many proteins in mitochondria, we and others have since sought out other mitochondrial proteins or components which may be at risk of nitrative/oxidative damage by peroxynitrite. These are complexes I, II, and V as well as ANT, creatine kinase, aconitase and manganese SOD (SOD2) (63). We intend to examine the nitration/oxidative modification of some of these compounds.

The free radical hypothesis of PD suggests that oxidative stress is implicated in the death of DA neurons in the SNpc (64). Several reactions within the DA neuron set the stage for this damaging situation. For instance, the reaction between the superoxide radical and NO puts cells in an oxidative/nitrative stress situation. Furthermore, the fact that the superoxide radical can be overproduced in several instances (Blockade of complex 1, induction and up-regulation of NADPH oxidase) also indicates oxidative stress. Therefore, the superoxide radical, by itself, can exert damaging effects by generating other reactive species such as the hydroxyl radical ($HO^{\cdot}$) whose oxidative properties can ultimately kill cells (5). Superoxide can facilitate $HO^{\cdot}$production in the metal-catylized Haber-Weiss reaction and can be dismutated by SOD1 to form hydrogen peroxide (H_2O_2) (5). In certain oxidative damage situations, there exists an oxidative pathway that does not require metal ions. This pathway involves myeloperoxidase, a heme protein secreted

by activated macrophages (51). Myeloperoxidase uses H_2O_2 to convert the phenolic acid tyrosine into a reactive intermediate that promotes the oxidation of proteins and lipids. Studies indicate that the oxidizing intermediate generated by myeloperoxidase is the tyrosyl radical (51) and suggest that this radical may promote oxidative reactions at sites of inflammation. Stable endproducts of protein oxidation act as indicators that a specific reaction has occurred thus we used gas chromatography with mass spectrometry to measure these reactions first in an *in vitro* situation with myeloperoxidase to determine what these reaction products might be, then *in vivo* following MPTP administration reaction to see if they were indeed part of the MPTP neurotoxic process. We found elevated levels of 3-nitrotyrosine and o,o-dityrosine in the ventral midbrains and striata of MPTP-treated mice as compared to saline control tissues. Cerebellum and frontal cortex showed no evidence of protein oxidation (65). The presence of these compounds in the brain indicate that oxidative modification of proteins took place in brain areas sensitive to the effects of MPTP and the reaction in the test tube confirms that a myeloperoxidase-H_2O_2 oxidative pathway is responsible for the existence of the oxidized proteins found in the brain tissues. These findings give further support to the oxidative stress hypothesis of PD and may be relevant to our understanding of the pathogenesis of this debilitating disorder.

We have put together a series of experiments to delve deeper into the mysteries of PD by examining the role of NO in the MPTP neurotoxic process. Our proposal is largely based on the free radical hypothesis (oxidative stress) of PD. From these studies, we conclude that the superoxide radical and NO are indeed instrumental in the death of DA neurons in the SNpc of MPTP-treated mice and are probably involved in the production and progression of PD. Production of oxidative stress is both internal and external to the DA neuron and apparently involves induction of iNOS and the up-regulation of nNOS, production of the superoxide radical, microglial activation with all of its cytotoxic components, peroxynitrite production and protein oxidation and nitration. A number of these same findings have also been found in PD, the human condition that MPTP mimics. Our findings suggest that PD is indeed a multifactorial disorder in which NO seems to play a major role. Thus, therapy may require the use of several drugs simultaneously to treat its symptoms.

APPENDICES (SEE ENCLOSED MANUSCRIPTS AND FIGURES for eNOS STUDIES).

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REVIEWS

TARGETING PROGRAMMED CELL DEATH IN NEURODEGENERATIVE DISEASES

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Molecular pathways of programmed cell death (PCD) are activated in various neurodegenerative disorders including Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease. In these diseases, PCD might be pathogenic, and targeting it might mitigate neurodegeneration. To identify potential neuroprotective targets within the PCD machinery, the expression and activity of some of its components have been altered by genetic or pharmacological means in experimental models of neurodegenerative diseases. The results of these studies have provided leads for the development of neuroprotective strategies for these progressive, disabling and often fatal disorders.

NEUROLOGICAL DISEASES

TUNEL TECHNIQUE
This technique enables the visualization of cells undergoing apoptosis by labelling the broken ends of the double-stranded DNA with biotin-conjugated dUTP, using the enzyme terminal deoxynucleotidyl transferase.

Programmed cell death (PCD) is a physiological process in which molecular programs that are intrinsic to the cell are activated to cause its destruction. This process is a fundamental property of all multicellular organisms and is crucial for their development, for organ morphogenesis, for tissue homeostasis and for defense against infected or damaged cells. The importance of PCD is emphasized by its remarkable degree of evolutionary conservation. However, excessive PCD can cause unwarranted cell death, which might lead to diseases such as immunodeficiency and neurodegeneration.

The term PCD is often used interchangeably with 'apoptosis' — a morphological form of cell death that is characterized by membrane blebbing, shrinkage of the cell body, nuclear condensation and DNA fragmentation. However, apoptosis is only one morphological form of PCD[1]; the molecular pathways linked to PCD are implicated in cell-death processes, the morphological diversity of which extends beyond apoptosis[2,3]. At times, even necrosis, which is traditionally considered to be a 'passive' death process (that is, death that does not rely on intracellular signalling pathways), has been prevented by anti-PCD compounds[4]. In this article, we group under the term PCD (also sometimes referred to as 'active cell death'[5]) all cell death forms that involve active intracellular processes, and use 'apoptosis' only in reference to the morphology of dying cells.

Over the past ten years, three experimental waves have characterized the study of PCD in neurodegeneration. Initially, the focus was on the search for apoptotic cells in *post mortem* tissues. This effort was undertaken in relation to several neurodegenerative disorders and gave rise to conflicting results. One lesson learned from these investigations was that looking for apoptosis in *post mortem* human tissue is complicated by many conceptual and technical factors. First, it is difficult to detect apoptosis owing to the presumed low daily rate of neuron loss in neurodegenerative disorders and the presumed rapid disappearance of apoptotic cells. Second, *post mortem* specimens typically derive from advanced stages of the disease, when most of the neurons that are affected by the pathological process are already lost. Third, most morphological *post mortem* studies rely on the so-called terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) TECHNIQUE to document the presence of apoptotic cells. However, we now know that TUNEL is not specific for apoptosis, especially in human *post mortem* tissue, in which factors such as hypoxia can produce TUNEL-positive, non-apoptotic DNA damage[6].

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Box 1 | **Programmed cell death and Alzheimer's disease**

Amyloid-β (Aβ) — a peptide that accumulates in the brains of people with Alzheimer's disease (AD) and forms amyloid plaques — directly induces apoptosis of cultured neurons[143]. Therefore, many researchers have looked for signs of programmed cell death (PCD) in Alzheimer patients. Along this line, fragmentation of nuclear DNA, detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)[144], has been detected in brains of people with AD, although this technique lacks specificity. More specific biochemical evidence that PCD might occur in AD is provided by the detection of activated caspases 3, 8 and 9 in hippocampal neurons of brains affected by AD[145–148]. Moreover, pharmacological or molecular inhibition of particular members of the caspase family, such as caspases 2, 3, 8 and 12, has been reported to offer partial or complete protection against Aβ-induced apoptotic cell death *in vitro*[25,149–151]. Aβ is derived from γ-secretase-mediated processing of the amyloid precursor protein (APP), but it has been shown, in both cultured cells and brains affected by AD, that APP can also be cleaved by caspases, such as caspase 3, at sites distinct from the classic secretase-processing sites[152]. Caspase-mediated cleavage of APP not only releases Aβ, but can also release a carboxy-terminal peptide that is a potent inducer of apoptosis[153]. Similarly, caspase 3-cleaved fragments of tau, a microtubule-associated protein that is the primary protein component of the filaments found in the brains of people with AD, have also been detected in *post mortem* samples[148]. Despite all of these data, there is no evidence of caspase activation or apoptotic cell death in animal models of AD and, therefore, there is no evidence *in vivo* for a potential beneficial effect of blocking PCD pathways in AD.

Given these problems, many investigators have stopped using an exclusively morphological approach, and now include techniques that assess molecular components of the PCD machinery. Although this combined approach has often shed light on the state of PCD in neurodegenerative diseases, none of these *post mortem* findings have established a role for PCD in the pathogenic process.

More recently, investigators have lost interest in the demonstration of PCD-associated cellular and molecular changes in human tissues, and they now focus on showing the actual role of PCD in the neurodegenerative process. To achieve this goal, key PCD molecules have been manipulated (either in transgenic or knockout studies), or inhibited by pharmacological agents or viral vectors in experimental models of neurodegenerative disorders. These studies have not only identified PCD components that either promote or prevent neuronal death, but they have also disclosed molecular targets for the development of drugs for preventing and treating neurodegenerative disorders.

In this article, after discussing the molecular composition of the PCD machinery, we review the results of manipulating such molecular pathways on the fate of neurons in experimental models of three prominent neurodegenerative disorders: Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). Although some work has been done on PCD in Alzheimer's disease[7] (BOX 1), the benefit of targeting PCD in experimental models of Alzheimer's disease remains to be determined; so, we will not discuss Alzheimer's disease here. Similarly, we will not discuss PCD in stroke[8], as this disorder falls outside the scope of an article on neurodegenerative diseases[9].

ZYMOGENS
The inactive precursors of enzymes — often transformed into the active enzyme by partial proteolysis.

Molecular pathways of PCD

The key mediators of PCD are the proteolytic enzymes called 'caspases', which cleave their substrates after specific aspartic acid residues. Caspases exist as ZYMOGENS (procaspases) in almost every animal cell, and they become activated in response to intracellular signalling pathways that are triggered by various cellular perturbations, such as DNA damage and withdrawal of trophic support. The family of mammalian caspases comprises 15 members, which can be divided into initiators (procaspases 2, 8, 9 and 10) and effectors (procaspases 3, 6 and 7) of PCD.

The initiators are the first caspases to become activated in the PCD cascade. They have long amino-terminal prodomains that contain specific protein–protein interaction motifs. Through these domains, initiator caspases 8 and 9 are activated after being aggregated by the adaptor molecules FADD (Fas-associating protein with death domain)[10] and Apaf1 (apoptotic protease-activating factor 1)[11], respectively. On activation, initiator caspases can cleave effector procaspases into their active forms, which are responsible for events such as mitochondrial damage, nuclear membrane breakdown, DNA fragmentation, chromatin condensation and, eventually, cell death.

The Bcl2 family of proteins, which are implicated in the regulation of PCD, comprises members that have either anti-PCD (such as Bcl2 and Bcl-x_L) or pro-PCD (such as Bax and Bak) effects[12]. Structurally, they all share some degree of similarity and can have up to four Bcl2-homology domains (BH1–BH4). Besides the many Bcl2 members that contain BH domains, such as Bcl2 *per se* and Bax, there are molecules that share sequence homology only with the BH3 domain, such as Bid or Bim. These BH3-only proteins can act as intracellular death ligands, proximal to multidomain Bcl2 members, and can connect with proximal signal transduction pathways[13]. Multidomain Bcl2 members can preserve or disrupt mitochondrial integrity by regulating the release of mitochondrial apoptogenic factors such as cytochrome *c*, Smac/Diablo or apoptosis-inducing factor (AIF). Bcl2 can also inhibit initiator caspases by a mitochondrial-independent mechanism[14].

The death receptor (or extrinsic) PCD pathway. The extrinsic PCD pathway (FIG. 1) is recruited on activation of cell-surface death receptors such as Fas/CD95 and the tumour necrosis factor receptor 1 (TNFR1). Death-receptor activation is initiated by specific ligands called death activators — the Fas ligand binds to Fas, and TNFα binds to TNFR1. On binding, the intracellular 'death domains' on these receptors associate with an adaptor protein that contains 'death effector domains', Fas associates with FADD, and TNFR1 associates with FADD and TRADD (TNFR-associated protein with death domain). Adaptor proteins then recruit procaspase 8, leading to its activation. Activated caspase 8 can then activate other caspases, either directly or indirectly, by cleaving Bid (FIG. 1). The extrinsic PCD pathway is especially instrumental in pathological conditions in which inflammation is a prominent feature. Because there is a growing appreciation that inflammation is a

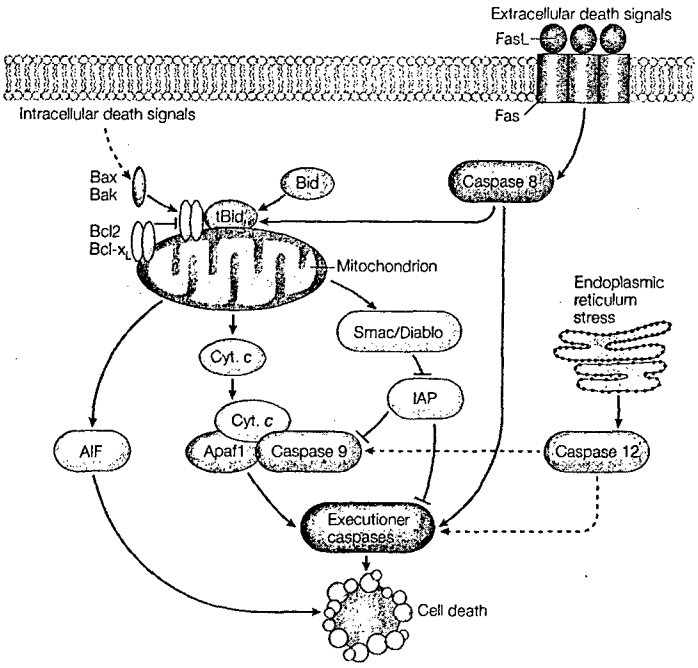


Figure 1 | **Molecular pathways of programmed cell death.** Extracellular signals through cellular death receptors, such as Fas, and intracellular signals, including damage to subcellular constituents or endoplasmic reticulum stress, both trigger genetically programmed pathways of programmed cell death (PCD). The two main PCD pathways result in the activation of downstream executioner caspases and cell death. AIF, apoptosis-inducing factor; Cyt., cytochrome; FasL, Fas ligand; IAP, inhibitor of apoptosis; tBid, truncated Bid.

feature of neurodegeneration with potential pathogenic significance[15], targeting the extrinsic PCD pathway in neurodegenerative diseases is a warranted approach. In addition to being activated by the extrinsic pathway, procaspase 8 also seems to be cleaved by a mechanism that depends on the intrinsic pathway[16].

The mitochondrial (or intrinsic) PCD pathway. In the intrinsic PCD pathway, death receptor-independent stimuli can trigger PCD by inducing translocation of pro-PCD molecules (such as Bax) to the mitochondria, with the subsequent release of mitochondrial apoptogenic factors (such as cytochrome *c*) to the cytosol (FIG. 1). Once released from the mitochondria, cytochrome *c* interacts with two other cytosolic protein factors[17], Apaf1 and procaspase 9, to activate caspase 3. The formation of this multimeric Apaf1–cytochrome *c* complex might serve to increase the local concentration of procaspases for intermolecular cleavage, and to set a relatively high threshold of caspase activation so that an occasional leakage of cytochrome *c* will not cause cells to undergo PCD[17]. Smac/Diablo is another mitochondrial intermembrane protein that is released into the cytosol on induction of PCD[18,19]. Once in the cytosol, Smac/Diablo interacts with several inhibitors of apoptosis (IAPs), therefore relieving the inhibitory effect of IAPs on initiator (such as caspase 9) and effector caspases (such as caspase 3)[20,21]. In contrast to cytochrome *c* and Smac/Diablo, the release of AIF and endonuclease G from the mitochondrial intermembrane space does not lead to caspase activation[22,23]. Under certain death-inducing stimuli, AIF translocates from the mitochondria to the nucleus where it induces caspase-independent, large-scale DNA fragmentation[22]. Similarly, endonuclease G, which is normally involved in the replication of mitochondrial DNA, can translocate to the nucleus on induction of PCD, and can induce fragmentation of nuclear DNA[23]. It has been reported that wah-1, the AIF homologue in *Caenorhabditis elegans*, associates and cooperates with endonuclease G to promote DNA degradation and apoptosis[24].

Stress in the endoplasmic reticulum (ER), including the disruption of calcium homeostasis and accumulation of unfolded proteins in the ER, can also result in PCD[25] through activation of caspase 12. Active caspase 12 can, in turn, cleave caspase 9 (REF. 26). Pro-PCD members of the Bcl2 family — such as Bax and Bak — operate at the ER to maintain calcium homeostasis and regulate ER-dependent PCD[27].

Targeting PCD in Parkinson's disease

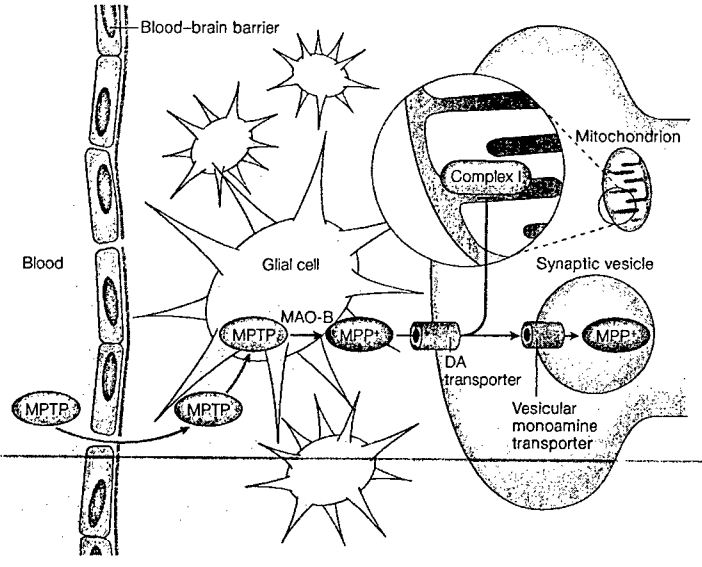
Parkinson's disease (PD) is a common neurodegenerative disorder of unknown cause, characterized by resting tremor, slowness of movement, rigidity and postural instability[28]. PD symptoms are attributed to the loss of dopamine (DA)-containing neurons in the substantia nigra pars compacta (SNPC). In the United States alone, this disease affects about one million people[28]. Although several approved drugs alleviate PD symptoms, none of them seem to stop or slow the neurodegenerative process.

Initially, the demonstration of increased numbers of TUNEL-positive DA neurons in the brains of patients with PD has been used to support the occurrence of apoptosis in this disease[29]. Subsequent studies, using a greater variety of morphological tools, have either succeeded[30–32] or failed[33–35] to find more apoptotic neurons in *post mortem* tissue from PD patients, leading to lively discussions in the field about whether apoptosis in PD is a myth or a reality. Moving away from morphological assessments, immunolocalization of Bax shows that a greater percentage of DA neurons in the SNPC were positive for this pro-PCD protein in brains of patients with PD as compared with controls[36]. In addition, Bax content seemed higher in the remaining DA neurons[37], consistent with an ongoing neurodegenerative process. DA neurons with increased expression and subcellular redistribution of the anti-PCD protein Bcl-x_L, and with increased activity of the effector protease caspase 3, have been found in greater abundance in the SNPC of people with PD as compared with controls[38,39], although these findings could not be independently confirmed[35]. Other PCD-related alterations detected in the brain of patients with PD include the activation of caspase 8 (REFS 40,41) and caspase 9 (REF. 41). But despite this body of descriptive data, the evidence cannot be regarded as an unequivocal demonstration that PCD has a pathogenic role in PD.

To address this crucial issue, it is necessary to use experimental models that allow manipulations of the PCD machinery and to assess their impact on neuronal

Box 2 | **The MPTP model of Parkinson's disease**

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analogue with potent heroin-like effects that can induce a parkinsonian syndrome in humans almost indistinguishable from Parkinson's disease (PD)[154]. Since the discovery that MPTP causes parkinsonism in humans and non-human primates, as well as in various other mammalian species, it has been used extensively as a model of PD. From neuropathological data, MPTP administration causes damage to the nigrostriatal dopamine (DA) pathway identical to that seen in PD, with the exception of the intraneuronal inclusions known as Lewy bodies. It is worthwhile noting that *post mortem* brain samples from patients with PD show a selective defect in the mitochondrial electron transport chain complex that is affected by MPTP[155,156]. The metabolism of MPTP is a complex, multistep process (see figure). After its systemic administration, MPTP, which is a pro-toxin, rapidly crosses the blood–brain barrier and is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine oxidase B (MAO-B) in non-DA cells, and then, probably by spontaneous oxidation, to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. MPP^+ is then taken up by DA transporters, for which it has high affinity. Once inside DA neurons, MPP^+ is concentrated by an active process within the mitochondria, where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of free radicals, which causes oxidative stress and activation of programmed cell death molecular pathways.



death. For the past twenty years, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD has generated important insights into PD pathogenesis and the role of PCD in PD (BOX 2). In addition to the MPTP model, the chronic infusion of rotenone to rats, which recapitulates most of the pathological hallmarks of PD[42], is emerging as an invaluable model to investigate PD pathogenesis. But compared to MPTP, the rotenone model is technically challenging and might not be as specific for the SNPC DA neurons[43] as initally claimed[42]. Genetic models of PD, based on the overexpression of α-synuclein, have also been developed (see later discussion)[44–48], but the lack of consistent SNPC DA neurodegeneration makes these models more suitable for studying pathogenic processes other than DA neuronal death.

Alterations in energy metabolism, generation of reactive oxygen species (ROS) and perturbations in calcium homeostasis occur within hours of MPTP administration — well before any significant neuronal death has occurred[49] — raising the possibility that these events are not the main effectors of cell death. Instead, they could be intracellular signals that can set into motion deleterious molecular cascades — such as PCD activation — which are ultimately responsible for the demise of DA neurons. The prolonged administration of relatively low doses of MPTP to mice leads to morphologically defined apoptotic DA neurons[50]. Under this regime of MPTP intoxication, Bax is strongly upregulated in SNPC DA neurons[51], whereas Bcl2 is downregulated. In this model, activation of Bax induces the recruitment of the mitochondrial PCD pathway, with the subsequent activation of caspase 9 and caspase 3 (REFS 38,41). The key role of Bax in MPTP-induced neurotoxicity is illustrated by the demonstration that mutant mice deficient in Bax are resistant to the toxicity of MPTP[51]. Overexpression of *Bcl2* also protects DA cells against MPTP-induced neurodegeneration[52,53].

How the deregulation of Bcl2 family members occurs after MPTP administration is unknown. It is improbable that MPTP directly alters Bax expression and conformation. Instead, it is more plausible that MPTP activates intracellular signalling pathways, which, in turn, cause Bax upregulation and its post-translational activation (for example, Bax oligomerization and internalization into mitochondrial membranes). The tumour supressor protein p53 is one of the rare molecules known to regulate *Bax* expression[54], and p53 is activated after MPTP intoxication[55], probably in response to MPTP-induced DNA damage[56]. Inhibition of p53 attenuates MPTP-induced Bax upregulation and the degeneration of DA neurons[57]. In addition, p53 null mice are resistant to the MPTP-induced death of DA neurons[58]. Activation of the Jun N-terminal kinase (JNK) pathway has also been observed after MPTP administration[59,60]. Moreover, pharmacological blockade of JNK activation with CEP-1347/KT-7515 (REF 61) or CEP-11004, or its inhibition by adenoviral gene transfer of the JNK-binding domain of JNK-interacting protein 1 (REF. 60) resulted in a marked attenuation of MPTP-induced neurodegeneration. *In vitro* evidence indicates that JNK activation caused by DNA damage is required for the mitochondrial translocation of Bax and the resulting recruitment of the mitochondrial PCD pathway[62,63]. These data indicate that, in the MPTP mouse model of PD, both p53 and JNK might act in concert to cause Bax induction and the post-translational changes that are mandatory to its pro-PCD role.

The intrinsic PCD pathway is recruited after MPTP administration[41], and blockade of this PCD pathway by an intrastriatal injection of an adeno-associated viral (AAV) vector containing a DOMINANT-NEGATIVE form of Apaf1 prevents the MPTP-induced activation of caspase 3 and the ensuing SNPC neuronal death[64]. By contrast, data on the recruitment and the importance of the extrinsic PCD pathway in the MPTP mouse model is still lacking.

DOMINANT NEGATIVE
A mutant molecule that can form a heteromeric complex with the normal molecule, knocking out the activity of the entire complex.

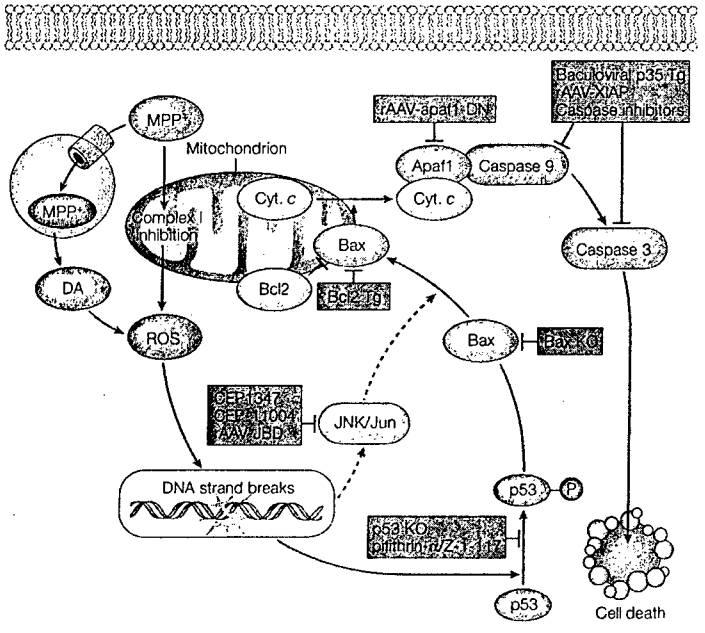


Figure 2 | **Proposed mechanism of MPTP-induced programmed cell death.** 1-methyl-4-phenylpyridinium (MPP^+), the toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), impairs mitochondrial respiration in dopaminergic neurons by inhibiting complex I of the electron transport chain. Inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of reactive oxygen species (ROS). MPP^+ can also redistribute vesicular dopamine (DA) to the cytosol. There, DA readily auto-oxidizes, thereby generating ROS. Both mitochondrial and cytolsolic MPP^+-related ROS produtions damage cellular elements, including DNA, and probably alter the expression of redox-sensitive transcription factors. ROS and damaged DNA activate p53, which induces upregulation of Bax. Bax is subsequently translocated into the mitochondria, probably by mediation of Jun N-terminal kinase (JNK), where it induces the release of cytochrome (cyt.) *c* to the cytosol and the ensuing caspase activation and cell death. Approaches aimed at targeting different key elements of this cascade (red boxes) result in an attenuation of MPTP-induced neurodegeneration. DN, dominant-negative; JBD, JNK-binding domain; KO, knockout; rAAV, adeno-associated virus vector delivery; tBid, truncated bid; Tg, transgenic, XIAP; X-chromosome-linked inhibitor of apoptosis.

Approaches that are aimed at inhibiting PCD at the level of the effector caspases have given inconsistent results. Adenoviral gene transfer of X-chromosome-linked inhibitor of apoptosis (XIAP) — a protein caspase inhibitor — prevents MPTP-induced SNPC neuronal death, but does not prevent the loss of striatal DA nerve fibres[65]. By contrast, transgenic mice overexpressing p35 — a general caspase inhibitor — in neurons showed attenuated MPTP-induced DA cell death and striatal DA depletion[41]. The discrepancy between these two studies might be explained by the more comprehensive inhibition of caspases mediated by p35 compared with XIAP, as p35 not only inhibits executioner caspases (such as caspases 3 and 7), but also upstream initiator caspases, such as caspases 8 and 9. In both studies, however, DA cell death was assessed early after MPTP administration, raising the question of whether the obtained effect indicates an actual protection or merely a delay in the cell death process. Similar to the findings with XIAP, some *in vitro* studies indicate that it is possible to produce resistance to cell death selectively at the level of the cell body. The broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk), and peptide inhibitors of caspases 2, 3 and 9 attenuate the loss of DA-containing ventral midbrain cell bodies (but not neurites) exposed to the active metabolite of MPTP — 1-methyl-4-phenylpyridinium (MPP^+) — in culture[66]. This observation indicates that the molecular pathways that lead to the destruction of the soma might differ from those that govern axonal degeneration[67]. In another study[40], zVAD-fmk failed to protect cultured ventral midbrain neurons from the effect of MPP^+, but instead triggered a morphological switch from apoptosis to necrosis. Minocycline, a clinically approved tetracycline derivative that inhibits microglial activation and presumably also inhibits caspase 1 and caspase 3 (REF. 68), mitigates MPTP-induced neurodegeneration[69,70]. Caspase 8, an initiator caspase in the extrinsic PCD pathway, is also activated in MPTP-treated mice and in patients with PD[40,41]. Paradoxically, activation of caspase 8 in the MPTP model occurs after the recruitment of the intrinsic, and not the extrinsic, PCD pathway[41], indicating that caspase 8 might participate in the amplification, rather than in the initiation of the PCD in SNPC DA neurons.

Another molecule that has been linked to neurodegeneration in PD is α-synuclein, mutations of which cause a familial form of PD[71,72]. α-Synuclein is also an important component of the neuropathological hallmark of the disease — the intracellular inclusions known as Lewy bodies[73]. In MPTP-treated animals, α-synuclein accumulates and becomes nitrated in the cytosol of SNPC DA neurons[74,75]. Ablation of α-synuclein in mice prevents MPTP-induced neurodegeneration[76]. Although a direct link between α-synuclein and DA cell death is not yet well established, expression of mutant α-synuclein in cell cultures promotes apoptosis[77], and cytochrome *c* stimulates the aggregation of α-synuclein *in vitro*[78,79]. Other pathways might also be involved in triggering PCD in PD. For example, in the substantia nigra from patients with PD, the levels of the antioxidant glutathione are reduced[80] — an event that can induce PCD in neurons[81].

Together, these data indicate that the molecular pathways of PCD are involved in the death of SNPC DA neurons in experimental models of PD (FIG. 2). They support the contention that alterations in the components of the PCD machinery that have been identified in the brain of people with PD are of pathological importance. It is noteworthy that targeting PCD upstream of its execution phase results in a marked attenuation of neurodegeneration, whereas interfering at a more downstream level, such as caspase activation, produces variable results. This is of therapeutic relevance because, once the caspase executioner program is in place, its inhibition might only delay cell death. As the symptoms of PD are caused by the loss of DA terminals in the striatum, preventing the death of SNPC cell bodies without preventing the degeneration of their axons is unlikely to be a helpful therapeutic strategy. Neurons might have two self-destruction programmes, one for PCD in the cell body and a second

Box 3 | The transgenic *SOD1* model of amyotrophic lateral sclerosis

After the discovery that mutations in the gene that encodes the cytosolic free radical scavenging enzyme copper/zinc superoxide dismutase (SOD1) are responsible for the familial form of amyotrophic lateral sclerosis (ALS)[157,158], it has been shown that the transgenic expression of different *SOD1* mutants in mice[83–85] and rats[86] replicates the clinical and pathological hallmarks of ALS. The age at onset of symptoms and the life span of these transgenic animals varies across different lines, depending on the mutation and its level of expression, but when symptoms appear, they invariably include motor abnormalities that progress with the same pattern as ALS[84,159]. The first motor abnormality in mice is a fine tremor, in at least one limb, when the animal is held in the air by the tail[159]. Thereafter, weakness and atrophy of proximal muscles, predominating in the hindlimbs, develop progressively. At the end-stage of the disease (~140 days), mice transgenic for mutant SOD1 (G93A) are severely paralyzed and can no longer feed or drink on their own[159]. These end-stage mice have a profound loss of motor neurons (~50%), many dystrophic neurites, marked gliosis, some Lewy body-like intracellular inclusions and motor neurons filled with phosphorylated neurofilaments[83,159–162].

one for the axon[67]. So, a combination of anti-PCD strategies might be required to obtain optimal clinical benefit from such neuroprotective approaches.

Targeting PCD in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a relentless, fatal disorder that is characterized by a loss of the motor neurons in the cerebral cortex and spinal cord[82]. The progressive decline of muscular function results in paralysis, speech and swallowing disabilities, emotional disturbance and, ultimately, respiratory failure, causing death within 2–5 years after the onset of the disease. So far, only a few approved treatments (such as mechanical ventilation and riluzole) prolong survival in patients with ALS to some extent.

Whereas about 90% of ALS cases are sporadic (they show no genetic linkage), about 10% are inherited[82]. Of these, about a fifth carry a dominant mutation in superoxide dismutase 1 (*SOD1*). Transgenic expression of different human ALS-linked *SOD1* mutations in mice[83–85] and rats[86] replicates the clinical and pathological hallmarks of ALS (BOX 3), regardless of whether the free-radical scavenging activity of SOD1 is increased, normal or almost absent[83–87]. This observation, combined with the fact that mutant mice deficient in SOD1 do not develop any motor neuron disease[88], indicates that the cytotoxicity of mutant SOD1 is a GAIN-OF-FUNCTION effect[89]. Transfected neuronal cells that express mutant SOD1 cDNA undergo apoptosis[90], as do PC-12 cells transfected with mutant SOD1 (REF. 91) and primary neurons from transgenic mice that express mutant SOD1 (REF. 92). These *in vitro* data led investigators to consider whether mutant SOD1 kills motor neurons by activating PCD. However, inhibition of PCD in transgenic mice that express mutant SOD1 delays, but does not permanently prevent, neurodegeneration[93,94]. This indicates that the recruitment of PCD in this experimental model of ALS does not result from a direct effect, but from an indirect effect of the mutant protein on the PCD machinery[95]. In keeping with this view, mutant SOD1 has the propensity to form intracellular aggregates, the presence of which, in the cytosol of motor neurons, might impair microtubule-dependent axonal transport[96]. It might therefore be possible that mutant SOD1, by stimulating protein aggregation, causes important motor neuron perturbations that, in turn, trigger PCD.

Although there are numerous reports on the neuropathological changes in the spinal cord of people with ALS, only a handful provide fine morphological descriptions of the dying motor neurons[97,98]. Some degenerating neurons have some features reminiscent of apoptosis, but none of these dying neurons can confidently be labelled as apoptotic. In mice transgenic for mutant SOD1, clear apoptotic cells are seen in the spinal cord, but are they are rare[99] and can be immunoreactive for neurofilament or glial fibrillary acid protein[100], indicating that both neuronal and glial cells are dying by apoptosis in this model. Non-apoptotic PCD variants, such as PARAPTOSIS[3] also seem to occur in this animal model of ALS[101]. The search for other markers of apoptosis in ALS has generated conflicting results. In one *post mortem* study, DNA fragmentation was detected by TUNEL in the spinal cord motor neurons from patients with ALS, but not from controls[102]. In two other studies, DNA fragmentation was detected not only in the motor cortex and spinal cord of people with ALS, but also in controls, although to a lesser degree[103,104]. In a subsequent study, internucleosomal DNA fragmentation was detected in the motor cortex and spinal cord of patients with ALS, but not in spared regions, such as the somatosensory cortex[98]. Here, DNA fragmentation was documented in the anterior horn of the spinal cord and in the motor cortex of patients by gel electrophoresis[98] — a highly specific technique that is not frequently used to identify apoptosis in the nervous system because of its lack of sensitivity. In contrast to these positive findings, other investigators, using similar techniques and tissue samples, have failed to provide any evidence of internucleosomal cleavage of DNA in *post mortem* tissue from patients with ALS or from animal models of the disease[105,106]. Two other apoptotic markers, the Le^y antigen and fractin, are highly expressed in the spinal cord of people with ALS[102] and transgenic *SOD1* mice[99], respectively, but neither marker has been detected in control subjects. Likewise, the levels of the PCD-related protein Par-4 (prostate apoptosis response-4) were increased in the spinal cord of patients with ALS and in mice transgenic for mutant SOD1, compared with their respective controls[107].

The study of known molecular mediators of PCD, irrespective of the morphology of the dying cells, in *post mortem* ALS samples and in mice transgenic for mutant SOD1 has provided more consistent results. In the lumbar cord of patients with ALS and transgenic *SOD1* mice, the mRNA content of *Bcl2* is decreased, whereas the level of *Bax* mRNA is increased as compared with controls[108,109]. At the protein level, spinal expression of Bcl2 and Bcl-x_L is either unchanged[110] or decreased[98,109], whereas that of Bax is increased[98,103,109] both in patients and *SOD1* mice. Because different *SOD1* mutations do not cause exactly the same neuropathology, it is important to stress that a similar pattern of changes of pro- and anti-PCD Bcl2 family members has been found in the

GAIN-OF-FUNCTION
A mutation that confers either a previously inexistent activity to the affected protein or increases a pre-existing funtion.

PARAPTOSIS
A form of programmed cell death that is related to apoptosis. It is transcription dependent and features swelling of the endoplasmic reticulum and mitochondria. However, it does not depend on caspase activation, except for caspase 9, lacks internucleosomal DNA cleavage, and does not show other morphological hallmarks of apoptosis such as nuclear fragmentation.

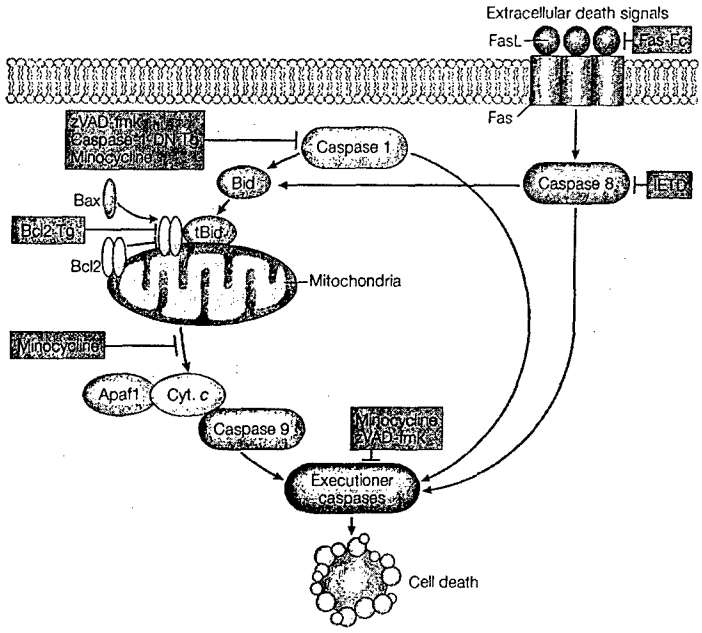


Figure 3 | **Targeting programmed cell death in amyotrophic lateral sclerosis.** In a transgenic mouse model of amyotrophic lateral sclerosis (ALS), initiator caspases activate downstream effector caspases mainly through the recruitment of the mitochondrial programmed cell death pathway. Interfering with different molecular elements of this cascade (red boxes), and particularly with caspase activation, significantly delays disease onset and mortality in this experimental model of ALS. Cyt., cytochrome; DN, dominant negative; FasL, Fas ligand; IETD Ile-Glu-Thr-Asp-fluoromethylketane ; tBid, truncated Bid; Tg, transgenic; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

spinal cords of another line of mice transgenic for SOD1 that harbour a different *SOD1* mutation[111]. None of these alterations, however, are seen in young asymptomatic transgenic mice, but they become progressively more manifest as the neurodegenerative process evolves[109]. In the spinal cord of both patients and affected transgenic *SOD1* mice, Bax is not only upregulated, but it is also mainly expressed in its deleterious homodimeric conformation[98,109]. In mice, Bax is relocated from the cytosol to the mitochondria[98,112]. These data indicate that, in ALS, the finely tuned balance between cell death antagonists and agonists is skewed towards death. Consistent with this idea, overexpression of Bcl2 mitigates neurodegeneration in both *in vitro* and *in vivo* models of ALS[91,93] and prolongs survival in transgenic *SOD1* mice[93]. As in the MPTP model, Bax upregulation in ALS is associated with p53 activation[111,113]. However, in contrast to the MPTP model, targeting p53 did not confer any protection against mutant SOD1-mediated neurodegeneration[114,115].

The pro-PCD protein Bid is expressed in the spinal cord of transgenic *SOD1* mice and is cleaved into its most active form during the progression of the disease[116]. However, whereas cleaved Bid is seen in mice transgenic for mutant SOD1 as early as the beginning of symptoms, activated caspase 8, which is known to cleave Bid, is detected only at the end-stage of the disease[116]. This observation indicates that cleavage of Bid might occur in this ALS model by another mechanism, perhaps involving caspase 1 (REF. 116), the inhibition of which prolongs the life-span of these animals[117]. Evidence for a prominent recruitment of the mitochondrial PCD pathway (that is, release of cytochrome *c* from the mitochondria to the cytosol) has been found in the spinal cord of patients and transgenic *SOD1* mice[112]. Moreover, pharmacological inhibition of cytochrome *c* release delays disease onset and extends survival of transgenic *SOD1* mice[118]. Activation of effector caspases, such as caspases 3 and 7, has also been reported in the spinal cord of patients with ALS[98] and of transgenic *SOD1* mice in a time-dependent manner that parallels the progression of the neurodegenerative process[99,100]. An instrumental role for caspases in ALS-related neurodegeneration is evidenced by the demonstration that zVAD-fmk attenuates mutant SOD1-mediated cell death in transfected PC-12 cells[91] and in transgenic *SOD1* mice[94], resulting in a delayed disease onset and mortality in these animals[94]. It has also been reported that mRNA and protein levels of the intrinsic caspase inhibitor XIAP are decreased in the spinal cord of symptomatic *SOD1* mice, and that induction of XIAP *in vitro* rescues cells harbouring mutant SOD1 from death by inhibiting the activation of caspase 3 (REF. 119). Activation of caspase 8, which mediates the death receptor pathway triggered by Fas, has been implicated in ALS in cultured embryonic motor neurons[120]. In this study, apoptotic cell death induced by trophic factor deprivation was rescued by Fas-Fc, an antibody fragment that blocks interactions between Fas and FasL, and by the caspase-8 inhibitor Ile-Glu-Thr-Asp-fluoromethylketone (IETD-fmk)[120]. In the presence of neurotrophic factors, exogenous Fas activators such as soluble FasL or anti-Fas antibodies triggered apoptosis of half of the purified motor neurons that were blocked by IETD-fmk[120]. Motor neurons isolated from transgenic mice that overexpress different ALS-linked SOD1 mutants showed increased susceptibility to activation of the Fas-triggered death pathway[121]. However, *in vivo* studies in mice transgenic for mutant *SOD1* indicate that activation of caspase 8, like induction of TNFα (REF. 122), occurs in the spinal cord only near end-stages of the cell-death process[116]. This observation indicates that the extrinsic PCD pathway in this model might make a late contribution to the neurodegenerative process.

Together (FIG. 3), the data that we have reviewed here indicate that interfering with PCD delays neuronal death and prolongs survival in experimental models of ALS. Eventually, these mice die despite the different interventions that we have discussed, indicating that targeting the PCD cascade can slow the death process but cannot abrogate it.

Targeting PCD in Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by choreic involuntary movements that result from selective neuronal loss in the striatum and the cerebral cortex. HD is caused by expansions of the trinucleotide CAG in

the *huntingtin* gene, producing a protein containing polyglutamine repeats[123]. Transgenic mice that overexpress a fragment of human huntingtin with an extended polyglutamine region show reduced survival, intraneuronal aggregates and behavioural deficits similar to HD[124]. Of these transgenic mice lines, the R6/2 line has been the most thoroughly studied. The length of the polyglutamine repeat is inversely correlated with the age of disease onset. HD is fatal, with a mean survival after onset of 15–20 years, and, at present, there is no effective treatment.

Although it is not known how mutant huntingtin promotes cell death, a self-amplification cascade of caspase activation has been proposed to participate. In *post mortem* brain tissue from patients with HD, TUNEL-positive cells, the morphology of which is reminiscent of what is found in apoptosis, have been detected in the neostriatum[125,126]. There is also evidence of the activation of caspases 1 and 8 (REFS 127,128) in the brain of patients with HD. Activation of caspase 1 was assessed by determining the levels of one of its substrates, interleukin-1β, and this might therefore reflect an inflammatory response rather than an activation of PCD pathways. Also, it is not clear whether activation of caspase 1 occurred in neurons or other cell types.

In experimental models of HD, there is evidence for the activation of caspases 1 and 3 (REF. 127). Furthermore, administration of ZVAD-fmk[127] or minocycline[68] — which inhibit caspases 1 and 3 — delays disease progression and mortality in the R6/2 transgenic mouse model of HD. Expression of a dominant-negative mutant form of caspase 1 in R6/2 mice extends survival and delays the appearance of neuronal inclusions, receptor alterations and the onset of symptoms[127]. Transgenic mice that express the caspase 1 dominant-negative mutation are also more resistant to neurotoxins that have been used to model HD, such as malonate and 3-nitropropionic acid[129]. Dietary restriction suppresses activation of caspase 1, reduces brain atrophy and the formation of huntingtin aggregates, delays disease onset and increases survival in another transgenic mouse model of HD, produced by the expression of a human amino-terminal truncated huntingtin with 82 polyglutamine repeats (HD-N171-82Q)[130].

Although these findings indicate that caspases might be valuable targets for therapeutic intervention in HD, how mutant huntingtin triggers PCD remains an enigma. It has been shown[131] that expression of extended polyglutamine repeats *in vitro* can directly activate initiator and effector caspases, such as caspases 3, 8 and 9. Moreover, wild-type huntingtin can attenuate PCD in cultured cells by preventing activation of caspase 3 through inhibition of the processing and activity of caspase 9 (REF. 132). However, the relationship between expanded polyglutamine repeats and PCD pathways seems more complex because, in addition to triggering the activation of PCD, some polyglutamine-containing proteins are caspase substrates. It has been shown that caspase 3 can cleave wild-type and mutant huntingtin proteins *in vitro*, thereby generating truncated fragments[133–135]. Truncated fragments that contain expanded polyglutamine repeats show increased toxicity and propensity to aggregate, compared with the full-length protein[133,134,136]. Huntingtin fragments have been identified in the brain of people with HD[137–140], but also in controls, indicating that caspase-mediated cleavage of wild-type huntingtin might occur as a normal physiological event. However, it is thought that cleavage of mutant huntingtin would release fragments with the potential for increased toxicity and accumulation, owing to the presence of the expanded polyglutamine tract[140].

Caspase 8 is another caspase that might be involved in the pathogenesis of HD. This caspase is recruited to

Table 1 | ***In vivo* and *in vitro* approaches to attenuate neurodegeneration in experimental models by targeting programmed cell death**

Disease	Experimental model	Genetic manipulation	Viral vector delivery	Pharmacological inhibition
PD	MPTP-treated mice	Bax KO[51]	JNK binding domain of JIP-1 (REF. 60)	Pifithrin-α/Z-1-117 (p53)[57]
			CEP-1347/KT-7515 (JNK)[61]	
		Bcl2 Tg[52,53]		CEP-11004 (JNK)[163]
		p53 KO[58]	Apaf1 (REF. 64)	Minocycline (caspases1, 3, cyt. c)[69,70]
		Baculoviral p35 Tg[41]	XIAP[65]	
	Mesencephalic dopaminergic neurons in culture			zVAD-fmk/M-920 (REF. 66) zVDVAD-fmk (caspase 2)[66] zDEVD-fmk/M-725 (caspase 3)[66] zLEHD-fmk (caspase 9)[66]
ALS	Transgenic *SOD1* mice	Bcl2 Tg[93]		zVAD-fmk[94]
		Caspase 1-DN Tg[117]		Minocycline (caspase 1, 3, cyt. c)[118]
	Embryonic motor neurons in culture			IETD-fmk (caspase 8)[120] Fas-Fc (Fas-FasL)[120]
HD	Transgenic R6/2 mice	Caspase 1-DN Tg[127]		zVAD-fmk[127] Minocycline (caspase 1, 3, cyt. c)[68]
	Striatal neurons in culture	Wild-type huntingtin transfection[132]		

ALS, amyotrophic lateral sclerosis; cyt., cytochrome; DN, dominant-negative; FasL, Fas Ligand; HD, Huntington's disease; IETD, Ile-Glu-Thr-Asp-fluoromethylketane; JNK, Jun N-terminal kinase; JIP, JNK interacting protein-1; KO, knockout; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease ; SOD1, superoxide dismutase; Tg, transgenic; XIAP, X-chromosome-linked inhibitor of apoptosis; zDEVD-fmk, benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; zLEHD-fmk, benzyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; zVDVAD-fmk, benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone.

intracellular aggregates and is subsequently activated in neuronal cells that express an expanded polyglutamine repeat[128]. In this system, caspase inhibition prevents cell death but not inclusion formation[128]. Furthermore, activated caspase 8 has been detected in the insoluble fractions of brains of people with HD but not of controls, further supporting the idea that caspase 8 is recruited to aggregates and subsequently activated[128]. It has also been proposed that caspase 8 activation in HD is mediated by the formation of pro-PCD heterodimers between Hip1 (huntingtin interacting protein 1) and Hippi (Hip1 protein interactor), which is favoured by the disease-associated polyglutamine expansion[141]. This non-receptor-mediated pathway for activating caspase 8 might trigger PCD through components of the extrinsic cell-death pathway and contribute to neuronal death in HD.

Together, these data indicate a complex association between expanded polyglutamine reapeat-containing proteins and caspase activation, indicating that caspases might also be a useful target for therapeutic intervention in HD.

Conclusions and perspectives

The different studies that we have reviewed here indicate that key molecular components of PCD are recruited in PD, ALS and HD. Much of the data on the occurrence of PCD in these diseases has been obtained by studying *post mortem* human brain samples. However, the information on the temporal relationship between these molecular changes, and their importance in the pathologic cascade, emanates largely from the use of animal models. Although not all of the diseases that we have reviewed show identical PCD changes, two general conclusions can be formulated. First, activation of PCD molecular pathways is a consistent feature of neurodegeneration. Second, PCD is not the sole mediator of cell demise in these disorders, but is a key component within a coalition of deleterious mechanisms that are responsible for the degenerative process.

The question of whether PCD pathways are involved in the demise of neurons in neurodegenerative diseases is more than academic, as it has important implications for the rational development of therapeutic strategies (TABLE 1). However, although the molecular complexity of the PCD cascade potentially offers many opportunities for its modulation, such pathways contain few conventional drug targets, such as enzymes and receptors. Therefore, researchers are focusing on other strategies to affect the components of the PCD pathway[142]. One approach is the modulation of the expression of key molecular components of the PCD machinery by gene and antisense therapy, but these technologies still need further development. Also, the problem of whether PCD can be selectively modulated in a specific organ or cell type, without adverse effects on others, remains to be solved. This issue is especially important in the context of chronic processes such as neurodegeneration that would require a sustained anti-PCD treatment. This problem could eventually be solved by fusing anti-PCD molecules with other molecules that are tagged to be recognized by tissue-specific receptors or uptake systems (for example, DA transporters). Alternatively, the problem could be tackled by synthesizing anti-PCD molecules as inactive pro-molecules that would be enzymatically activated in specific tissues or cell types (for example, by tyrosine hydroxylase in DA neurons).

One of the few potential pharmacological approaches that could modulate PCD involves inhibitors of caspase activity. According to the data that we have reviewed here, caspase inhibition might indeed delay cell death in different experimental models of neurodegeneration. However, targeting PCD at such a downstream point seems insufficient to stop the degenerative process. Nevertheless, it should be possible to combine caspase inhibition with other treatments directed at upstream events, as we believe that activation of PCD in neurodegenerative diseases is not a primary event. Instead, we think it is a 'suicide' decision, taken by cells that are affected by disease-related abnormalities such as inflammation, mitochondrial dysfunction, oxidative stress, or misfolding and aggregation of proteins. Targeting PCD alone is therefore unlikely to be sufficient to obtain a beneficial therapeutic effect. Optimal neuroprotection might require administration of a pharmacological cocktail directed against several pathogenic events.

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Online links

DATABASES

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AIF | Apaf1 | Bak | Bax | Bcl | Bcl-x$_L$ | Bid | Bim | caspase 1 | caspase 3 | caspase 8 | caspase 9 | caspase 12 | FADD | Fas | Hip1 | Hippi | *huntingtin* | JNK | JNK-interacting protein 1 | p53 | Par-4 | Smac/Diablo | *SOD1* | α-synuclein | TNFR1 | Tnfα | TRADD | XIAP

OMIM: http://www.ncbi.nlm.nih.gov/Omim/

Alzheimer disease | amyotrophic lateral sclerosis | Huntington disease | Parkinson disease

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APPENDIX 2

The 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model

A Tool to Explore the Pathogenesis of Parkinson's Disease

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ABSTRACT: **Experimental models of dopaminergic neurodegeneration play a critical role in our quest to elucidate the cause of Parkinson's disease (PD). Despite the recent development of "genetic models" that have followed upon the discovery of mutations causing rare forms of familial PD, toxic models remain at the forefront when it comes to exploring the pathogenesis of sporadic PD. Among these, the model produced by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has a competitive advantage over all other toxic models because once this neurotoxin causes intoxication, it induces in humans a syndrome virtually identical to PD. For the past two decades, the complex pharmacology of MPTP and the key steps in the MPTP neurotoxic process have been identified. These molecular events can be classified into three groups: First, those implicated in the initiation of toxicity, which include energy failure due to ATP depletion and oxidative stress mediated by superoxide and nitric oxide; second, those recruited subsequently in response to the initial neuronal perturbations, which include elements of the molecular pathways of apoptosis such as Bax; and, third, those amplifying the neurodegenerative insult, which include various proinflammatory factors such as prostaglandins. Herein, these different contributing factors are reviewed, as is the sequence in which it is believed these factors are acting within the cascade of events responsible for the death of dopaminergic neurons in the MPTP model and in PD. How to target these factors to devise effective neuroprotective therapies for PD is also discussed.**

KEYWORDS: **apoptosis; cell death; nitric oxide; neurotoxicity; neurodegeneration; MPTP; Parkinson's disease (PD); reactive oxygen species; superoxide dismutase**

INTRODUCTION

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a byproduct of the chemical synthesis of a meperidine analog with potent heroin-like effects. MPTP can induce a parkinsonian syndrome in humans almost indistinguishable from Par-

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kinson's disease (PD).[1] Recognition of MPTP as a neurotoxin occurred early in 1982, when several young drug addicts mysteriously developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs that, unknown to anyone, were contaminated with MPTP.[2] In humans and nonhuman primates, depending on the regimen used, MPTP produces an irreversible and severe parkinsonian syndrome that replicates almost all of the features of PD; in nonhuman primates, however, a resting tremor characteristic of PD has been demonstrated convincingly only in the African green monkey.[3] It is believed that in PD the neurodegenerative process occurs over several years, while the most active phase of neurodegeneration is completed within a few days following MPTP administration.[4,5] However, recent data suggest that, following the main phase of neuronal death, MPTP-induced neurodegeneration may continue to progress "silently" over several decades, at least in humans intoxicated with MPTP.[6,7] Except for four cases,[7,8] no human pathological material has been available for study; thus, the comparison between PD and the MPTP model is limited largely to nonhuman primates.[9] Neuropathological data show that MPTP administration causes damage to the nigrostriatal dopaminergic pathway identical to that seen in PD,[10] yet there is a resemblance that goes beyond the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons. Like PD, MPTP causes a greater loss of dopaminergic neurons in the SNpc than in the ventral tegmental area[11,12] and, in monkeys treated with low doses of MPTP (but not in humans), a greater degeneration of dopaminergic nerve terminals in the putamen than in the caudate nucleus.[13,14] However, two typical neuropathologic features of PD have, until now, been lacking in the MPTP model. First, except for the SNpc, pigmented nuclei such as the locus coeruleus have been spared, according to most published reports. Second, the eosinophilic intraneuronal inclusions called Lewy bodies, so characteristic of PD, have not, thus far, been convincingly observed in MPTP-induced parkinsonism;[9] however, in MPTP-injected monkeys, intraneuronal inclusions reminiscent of Lewy bodies have been described.[15] Despite these imperfections, MPTP continues to be regarded as an excellent animal model of sporadic PD, and the belief is that studying MPTP toxic mechanisms will shed light on meaningful pathogenic mechanisms implicated in PD.

Over the years, MPTP has been used in a large variety of animal species, ranging from worms to mammals. To date, the most frequently used animals for MPTP studies have been monkeys, rats, and mice.[16] The administration of MPTP through a number of different routes using different dosing regimens has led to the development of several distinct models, each characterized by some unique behavioral and neuropathological features. Herein, we will restrict our discussion to mice, since they have emerged as the preferred animals to explore cellular and molecular alterations produced by MPTP, in part because lines of engineered animals that are so critical to these types of investigations are available only in mice.[17]

MPTP MODE OF ACTION

As illustrated in FIGURE 1, the metabolism of MPTP is a complex, multistep process.[18] After its systemic administration, MPTP, which is highly lypophilic, rapidly crosses the blood-brain barrier. Once in the brain, the protoxin MPTP is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) by the enzyme monoamine

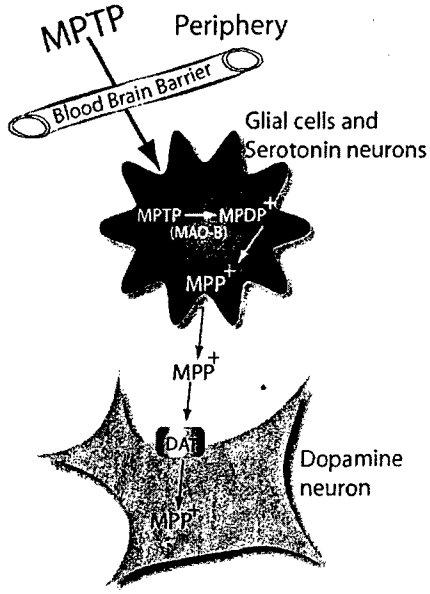

FIGURE 1. Schematic diagram of MPTP metabolism. After its systemic administration, MPTP crosses the blood–brain barrier. Once in the brain, MPTP is converted to $MPDP^+$ by monoamine oxidase B within nondopaminergic cells, and then to MPP^+ by an unknown mechanism. Thereafter, MPP^+ is released, again by an unknown mechanism, in the extracellular space. From there, MPP^+ is taken up by the dopamine transporter and thus enters dopaminergic neurons.

oxidase B within nondopaminergic cells, and then (probably by spontaneous oxidation) to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. Thereafter, MPP^+ is released (by an unknown mechanism) into the extracellular space. Since MPP^+ is a polar molecule, unlike its precursor MPTP, it cannot freely enter cells, but depends on the plasma membrane carriers to gain access to dopaminergic neurons. MPP^+ has a high affinity for plasma membrane dopamine transporter (DAT),[19] as well as for norepinephrine and serotonin transporters. The obligatory character of this step in the MPTP neurotoxic process is demonstrated by the fact that blockade of DAT by specific antagonists such as mazindol[20] or ablation of the DAT gene in mutant mice[21] completely prevents MPTP-induced toxicity. Conversely, transgenic mice with increased brain DAT expression are more sensitive to MPTP.[22]

Once inside dopaminergic neurons, MPP^+ can follow at least three routes (FIG. 2): (1) it can bind to the vesicular monoamine transporters (VMAT), which will translocate MPP^+ into synaptosomal vesicles;[23] (2) it can be concentrated within the mitochondria;[24] and (3) it can remain in the cytosol and interact with different cytosolic enzymes.[25] The fraction of MPP^+ destined to each of these routes is probably a function of MPP^+ intracellular concentration and affinity for VMAT, mitochondria carriers, and cytosolic enzymes. The importance of the vesicular sequestration of

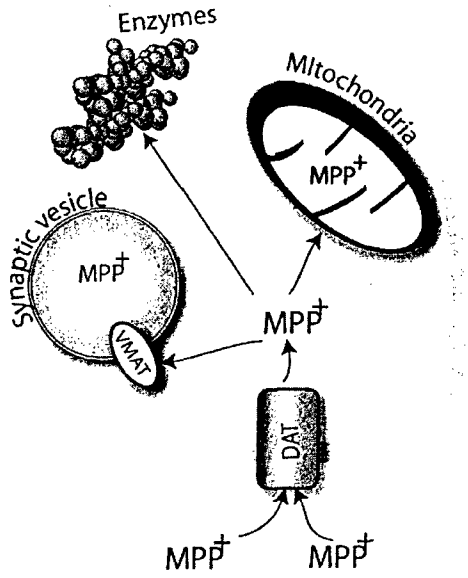

FIGURE 2. Schematic diagram of MPP^+ intracellular pathways. Inside dopaminergic neurons, MPP^+ can bind to the vesicular monoamine transporters, be translocated into synaptosomal vesicles, be concentrated by an active process within the mitochondria, and remain in the cytosol and interact with different cytosolic enzymes.

MPP^+ is demonstrated by the fact that cells transfected to express greater density of VMAT are converted from MPP^+-sensitive to MPP^+-resistant cells.[23] Conversely, we demonstrated that mutant mice with 50% lower VMAT expression are significantly more sensitive to MPTP-induced dopaminergic neurotoxicity compared to their wild-type littermates.[26] These findings indicate that there is a clear inverse relationship between the capacity of MPP^+ sequestration (that is, VMAT density) and the magnitude of MPTP neurotoxicity. Inside dopaminergic neurons, MPP^+ can also be concentrated within the mitochondria (FIG. 2),[24] where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain[27,28] through its binding at or near the site of the mitochondrial poison rotenone.[29,30]

MPTP MECHANISM OF ACTION

Currently, it is believed that the neurotoxic process of MPTP is made up of a cascade of deleterious events, which can be divided into early and late neuronal perturbations and secondary nonneuronal alterations. All of these, to a variable degree and at different stages of the degenerative process, participate in the ultimate demise of dopaminergic neurons.

Early Events

Soon after its entry into dopaminergic neurons, MPP^+ binds to complex I and, by interrupting the flow of electrons, leads to an acute deficit in ATP formation. It appears, however, that complex I activity must be reduced >70% to cause severe ATP depletion in nonsynaptic mitochondria[31] and that, in contrast to *in vitro* MPTP, *in vivo* MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels,[32] raising the question as to whether an MPP^+-related ATP deficit can be the sole factor underlying MPTP-induced dopaminergic neuronal death. Another consequence of complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS), especially of superoxide.[33–35] A recent demonstration[36] showed that early ROS production can also occur in this model from the autooxidation of dopamine resulting from an MPP^+-induced massive release of vesicular dopamine to the cytosol. The importance of MPP^+-related ROS production in the dopaminergic toxicity process *in vivo* is demonstrated by the fact that transgenic mice with increased brain activity of copper/zinc superoxide dismutase (SOD1), a key ROS-scavenging enzyme, are significantly more resistant to MPTP-induced dopaminergic toxicity than their nontransgenic littermates.[37] However, several lines of evidence support the concept that ROS exert many or most of their toxic effects in the MPTP model in conjunction with other reactive species such as nitric oxide (NO)[38–41] produced in the brain by both the neuronal and the inducible isoforms of the enzyme NO synthase.[42,43] Comprehensive reviews of the source and the role of NO in the MPTP model can be found in Przedborski and Vila[1] and in Przedborski and Dawson.[44]

Late Events

In response to the variety of functional perturbations caused by the depletion in ATP and the production of ROS, death signals, which can activate the molecular pathways of apoptosis, arise within intoxicated dopaminergic neurons. Although at this time we cannot exclude with certainty the possibility that apoptotic factors are in fact always recruited regardless of MPTP regimen, only prolonged administration of low-to-moderate doses of MPTP is associated with definite morphologically defined apoptotic neurons.[5,45] Supporting the implication of apoptotic molecular factors in the demise of dopaminergic neurons after MPTP administration is the demonstration that the proapoptotic protein Bax is instrumental in this toxic model.[46] Overexpression of the antiapoptotic Bcl-2 also protects dopaminergic cells against MPTP-induced neurodegeneration.[47,48] Similarly, adenovirus-mediated transgenic expression of the X chromosome–linked inhibitor of apoptosis protein (XIAP), an inhibitor of executioner caspases such as caspase-3, also blocks the death of dopaminergic neurons in the SNpc following the administration of MPTP.[49,50] Additional caspases are also activated in MPTP-intoxicated mice such as caspase-8, which is a proximal effector of the tumor necrosis factor receptor (TNFr) family death pathway.[51] Interestingly, however, in the MPTP mouse model it is possible that caspase-8 activation is consequent to the recruitment of the mitochondria-dependent apoptotic pathway and not, as in many other pathological settings, to the ligation of TNFr.[52] Other observations supporting a role of apoptosis in the MPTP neurotoxic process include the demonstration of the resistance to MPTP of the fol-

lowing: mutant mice deficient in p53,[53] a cell cycle control gene involved in programmed cell death; mice with pharmacological or genetic inhibition of c-Jun N terminal kinases;[54–56] or mice that received a striatal adenoassociated virus vector delivery of an Apaf-1–dominant negative inhibitor.[57] Collectively, these data show that during the degenerative process the apoptotic pathways are activated and contribute to the actual death of intoxicated neurons in the MPTP model.

Secondary Events

The loss of dopaminergic neurons in the MPTP mouse model is associated with a glial response composed mainly of activated microglial cells and, to a lesser extent, of reactive astrocytes.[58] From a neuropathological standpoint, microglial activation is indicative of an active, ongoing process of cell death. The presence of activated microglia in postmortem samples from MPTP-intoxicated individuals who came to autopsy several decades after being exposed to the toxin[59] suggests an ongoing degenerative process and thus challenges the notion that MPTP produces a "hit and run" kind of damage. Therefore, this important observation[59] suggests that a single acute insult to the SNpc by MPTP could set in motion a self-sustained cascade of events with long-lasting deleterious effects. With mice injected with MPTP and killed at different time points thereafter, it appears that the time course of reactive astrocyte formation parallels that of dopaminergic structure destruction in both the striatum and the SNpc, and that glial fibrillary acidic protein (GFAP) expression remains upregulated even after the main wave of neuronal death has passed.[60–62] These findings suggest that, in the MPTP mouse model,[63] the astrocyte activation is secondary to the death of neurons and not the reverse. This conclusion is supported by the demonstration that blockade of MPP^+ uptake into dopaminergic neurons completely prevents not only SNpc dopaminergic neuronal death but also GFAP upregulation.[64] Remarkably, activation of microglia, which is also quite strong in the MPTP mouse model,[60–62,65] occurs earlier than that of astrocytes and, more important, reaches a maximum before the peak of dopaminergic neurodegeneration.[62] In light of the MPTP data presented above, it can be surmised that the response of both astrocytes and microglial cells in the SNpc clearly occurs within a time frame allowing these glial cells to participate in the demise of dopaminergic neurons in the MPTP mouse model and possibly in PD. Activated microglial cells can produce a variety of noxious compounds, including ROS, reactive nitrogen species (RNS), proinflammatory cytokines, and prostaglandins. Observations showing that blockade of microglial activation mitigates nigrostriatal damage caused by MPTP supports the notion that microglia participate in MPTP-induced neurodegeneration.[66] Among specific deleterious factors, cyclooxygenase type-2 (Cox-2) has emerged as an important determinant of cytotoxicity associated with inflammation.[67,68] In the normal brain, Cox-2 is significantly expressed only in specific subsets of forebrain neurons that are primarily glutamatergic in nature,[69] which suggests a role for Cox-2 in the postsynaptic signaling of excitatory neurons. However, under pathological conditions, especially those associated with a glial response, Cox-2 expression in the brain can increase significantly, as does the level of its products (for example, prostaglandin E_2, or PGE_2), which are responsible for many of the cytotoxic effects of inflammation. Interestingly, Cox-2 promoter shares many features with inducible nitric oxide synthase (iNOS) promoter;[70] thus, these two enzymes are often coex-

pressed in disease states associated with gliosis. Therefore, it is not surprising to find Cox-2 and iNOS expressed in SNpc glial cells of postmortem PD samples;[71] PGE_2 content is also elevated in SNpc from PD patients.[72] Of relevance to the potential role of prostaglandin in the pathogenesis of PD is the demonstration that the pharmacological inhibition of both Cox-2 and Cox-1[73] and the genetic ablation of Cox-2 attenuates MPTP neurotoxicity.[74]

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APPENDIX 3

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Genetic clues to the pathogenesis of Parkinson's disease

Miquel Vila & Serge Przedborski

Recent years have seen an explosion in the rate of discovery of genetic defects linked to Parkinson's disease. These breakthroughs have not provided a direct explanation for the disease process. Nevertheless, they have helped transform Parkinson's disease research by providing tangible clues to the neurobiology of the disorder.

Parkinson's disease (PD) is the second most common human neurodegenerative disorder, after Alzheimer's dementia. This disease is progressive, with a mean age at onset of 55, and its incidence increases markedly with age[1]. The primary hallmark of PD is the degeneration of the nigrostriatal dopaminergic pathway, which, in depleting the brain of dopamine, initiates aberrant motor activity such as tremor at rest, rigidity, slowness of voluntary movement, and postural instability. As with other neurodegenerative disorders, however, the neuropathology of PD is not restricted to one pathway, and histological abnormalities also occur in many other dopaminergic and non-dopaminergic cell groups including the locus coeruleus, raphe nuclei and nucleus basalis of Meynert[2]. Because numerous distinct neurological conditions share the clinical features of PD, a definitive diagnosis of PD can only be made at autopsy, and it has customarily been based not only on the loss of nigrostriatal dopaminergic neurons but also on the presence of intraneuronal inclusions called Lewy bodies (LBs). These are spherical eosinophilic cytoplasmic aggregates containing a variety of proteins, of which α-synuclein is a major component[3], and are found in every affected brain region. Whether identification of LBs should still be considered necessary for the diagnosis of PD is controversial, in that individuals with inherited PD linked to mutations in the gene encoding parkin typically lack LBs and are still regarded as having PD[1]. Moreover, the role of LBs in the PD neurodegenerative process is a matter of fierce debate.

The cause of almost all cases of PD remains unknown. PD generally arises as a sporadic condition but is occasionally inherited as a simple mendelian trait (Table 1). Although sporadic and familial PD are very similar, inherited forms of the disease usually begin at earlier ages and are associated with atypical clinical features (Table 1). Until recently, all of the hypotheses regarding the cause and mechanism of PD neurodegeneration derived from investigations carried out on autopsy tissues from individuals with sporadic PD or in neurotoxic animal models such as that produced by the mitochondrial poison 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[1]. In the mid-1990s, however, this situation changed with the identification of a mutation in the α-synuclein gene associated with PD in an Italian kindred[4]. Since then, four additional genetic defects underlying PD have been identified and linkages have been reported for at least four more (Table 1).

Here we review what is currently known about these PD-causing mutations. As is the case in Alzheimer's disease, these gene defects seem to operate on a common molecular pathway. Thus, we also discuss this pathway and the directions in which those genes may lead us in regard to the development of genetically based animal models, which are crucial to unraveling the basis of the neurodegenerative processes of PD.

α-Synuclein mutations and dopaminergic neurodegeneration

Three missense mutations (A53T, A30P and E46K) in the gene encoding α-synuclein are linked to a dominantly inherited PD[4–6] (Table 1). None of these mutations has been found in sporadic PD or in individuals without the disease.

Injection of either human wild-type or mutant α-synuclein-expressing viral vectors into the rat and monkey nigrostriatal pathway causes dopaminergic neurodegeneration associated with α–synuclein-containing inclusions[7,8]. Transgenic overexpression of mutant or wild-type α-synuclein in mice or flies has produced equivocal results[1], however, in that intraneuronal proteinaceous inclusions, but not definite neuronal death, have generally been documented. Still, these results, together with the finding that α-synuclein ablation in mice does not cause neurodegeneration[9,10], support the notion that α-synuclein mutations operate by a toxic gain-of-function mechanism. Viral vector–mediated overexpression of wild-type α-synuclein reproduces PD neuropathology in animals[7,8], and genomic multiplication of the gene encoding α-synuclein is associated with a familial form of PD[11,12]. It is thus possible that the function gained by the mutant protein is not a newly acquired property, but rather a native property that is enhanced and becomes deleterious.

How mutant α-synuclein variants produce neurotoxicity remains elusive, in part because the protein's function is just beginning to be understood. Wild-type α-synuclein binds preferentially to plasma membranes (rather than mitochondrial membranes) in yeasts[13] and this interaction, which is mediated by major conformational changes of the protein[14], seems to be crucial to several of its physiological functions[1]. Membrane-bound α-synuclein has been proposed to modulate phospholipase D activity[15], thereby perhaps influencing the availability of synaptic vesicles for release. Membrane-bound α-synu-

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Table 1 Genes and loci linked to familial PD

Locus	Chromosomal location	Protein	Inheritance pattern	Atypical PD features	Lewy bodies
PARK1	4q21	α-Synuclein[a]	AD	Early onset Lower prevalence of tremor	Yes
PARK2	6q25.2–q27	Parkin	AR	Early or juvenile onset More frequent dystonia and levodopa-induced dyskinesias Slower disease progression	Mostly negative[b]
PARK3	2p13	Unknown	AD	Dementia in some individuals Rapid progression	Yes
PARK4[c]	4p15	Unknown	AD	Early onset Rapid progression Dementia Autonomic dysfunction Postural tremor	Yes
PARK5	4p14	UCH-L1	AD	None	Unknown
PARK6	1p36	PINK1	AR	Early onset Slow progression	Unknown
PARK7	1p36	DJ-1	AR	Early onset Psychiatric symptoms Slow progression	Unknown
PARK8	12p11.2–q13.1	Unknown	AD	None	No
PARK9	1p36	Unknown	AR	Juvenile onset Spasticity Supranuclear gaze paralysis Dementia	Unknown

AD, autosomal dominant; AR, autosomal recessive. [a]Including mutations and wild-type multiplications. [b]Lewy bodies reported in one individual with parkin mutations[54]. [c]The initial PARK4 linkage to 4p15 could not be confirmed, and the PD phenotype in this family was subsequently linked to a PARK1 variant (α-synuclein triplication)[11].

clein also seems to be in dynamic equilibrium with cytosolic α-synuclein[13], which upon accumulation can render endogenous dopamine toxic[16], and to act as a seed promoting the formation of cytosolic inclusions[17]. Conceivably if these aggregates are not promptly cleared by degradation pathways[18], neurotoxicity can ensue.

Both wild-type and mutant α-synuclein form amyloid fibrils akin to those seen in LBs, as well as nonfibrillary oligomers[1] termed protofibrils. Because the pathogenic α-synuclein^{A53T} mutant promotes the formation of protofibrils[19], these oligomers may be the toxic species of α-synuclein. In keeping with this and with the known association of α-synuclein with synaptosomes, protofibrils may cause toxicity by permeabilizing synaptic vesicles[20], allowing dopamine to leak into the cytoplasm and participate in reactions that generate oxidative stress. Furthermore, the selective vulnerability of nigrostriatal neurons in PD may derive from the ability of dopamine or dopamine-quinone to stabilize α-synuclein protofibrils[21]. However, protofibrils have only been identified and studied *in vitro*, and so further work is required to establish whether they form in neurons and whether their formation correlates with neurotoxicity.

Parkin, a protein with many substrates

Loss-of-function mutations in the gene encoding parkin cause a recessively inherited form of PD[22] (Table 1). The onset of parkin-related PD usually, but not always, occurs before age 30 (ref. 1). Pathologically, this form of familial PD is associated with a loss of nigrostriatal neurons, but LBs are not typically observed. Parkin-null mice and flies do not develop degeneration of nigrostriatal dopaminergic neurons[23–25]. However, these animals do show functional mitochondrial deficits[24–26] suggestive of those seen in sporadic PD[1].

Identification of the normal function of parkin has provided hints to the pathogenic effects of parkin mutations. Parkin is one of a class of proteins containing two RING-FINGER DOMAINS separated by an in-between RING-finger domain, and like other such proteins, parkin functions as an E3 ubiquitin ligase[27,28], a component of the ubiquitin system. Many mutations affecting parkin abolish its E3 ligase activity[1], as does the post-translational modification (*S*-nitrosylation) of wild-type parkin[29]. It is thus conceivable that parkin dysfunction is involved in the pathogenesis of both familial and sporadic PD, but the underlying molecular details remain speculative. Loss of parkin activity may trigger cell death by rendering neurons more susceptible to cytotoxic insults, such as those caused by proteasome inhibition or mutant α-synuclein[30], or by impairing ubiquitination of cyclin E[31], a molecule previously implicated in neuronal apoptosis. In support of the latter hypothesis, cyclin E is abundant in the midbrains of individuals with parkin-related PD, and overexpression of wild-type parkin attenuates cyclin E accumulation and promotes survival in excitotoxin-treated cultured neurons[31]. Several studies have shown functional interactions between parkin and α-synuclein, and have suggested that these interactions may involve the proteasome[1]. Other investigations have highlighted the multiplicity of parkin substrates and how these might have a key role in neuronal death[1]. However, none of the parkin substrates that have been identified seem to be specifically enriched in dopaminergic neurons. Thus, further studies are needed to explain the relative specificity of dopaminergic neurodegeneration mediated by parkin mutations.

UCH-L1 dabbles in degeneration

Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is expressed mainly in the brain, where it catalyzes the hydrolysis of C-terminal ubiquityl esters. A single dominant mutant form (I93M) of UCH-L1, found in two members of a PD-affected family, has been implicated in the development of an inherited form of PD[32]. Conversely, it has been confirmed that a polymorphism (S18Y) of UCH-L1 reduces the risk of developing sporadic PD, especially in early-onset cases[33]. The I93M mutation decreases the enzyme's activity, suggesting that a loss of function is the culprit in disease development. However, mice carrying a UCH-L1-null mutation do show neurodegenerative changes, but not in the nigrostriatal dopaminergic pathway[34]. Upon

dimerization, UCH-L1 can also exert an ubiquitin ligase activity, which is decreased by the pathogenic I93M mutation and increased by the protective S18Y polymorphism[35]. UCH-L1 was also identified as a protease with specificity for Nedd8 (ref. 36), a small ubiquitin-like protein implicated in cell-cycle regulation. Although all of these findings are consistent with the hypothesis that PD pathogenesis involves impairment of the ubiquitin systems, further studies are needed to elucidate how mutations affecting UCH-L1 kill dopaminergic neurons.

Multiplicity of DJ-1 mutations

DJ-1 is a homodimeric, multifunctional protein ubiquitously expressed in human tissues including the brain. Eleven different mutations affecting DJ-1—these include missense, truncating and splice-site mutations and large deletions—have been linked to an autosomal recessive form of PD[37–39]. Although DJ-1 mutations account for only a small fraction of early-onset PD, they are the second most frequent cause of recessive forms of PD after parkin mutations. These mutations are found throughout the *DJ-1* gene in four of its seven exons. This suggests that even though linked to a similar phenotype, the different mutations probably affect different domains of the protein with distinct functional and structural roles. This situation is reminiscent of that in amyotrophic lateral sclerosis, where the same paralytic syndrome is caused by many different mutations affecting superoxide dismutase-1 (http://alsod.org).

DJ-1 was discovered as part of a multiprotein complex that stabilizes mRNA through an interaction with *c-myc* and as a protein involved in infertility in rodents[40]. DJ-1 may modulate mRNA expression through interactions with a polypeptide complex comprising the androgen receptor and the SUMOYLATION enzyme PIASx[41,42]. DJ-1 may also function both as a sensor for oxidative stress and as an antioxidant[40,43]. In addition, structural studies indicate that DJ-1 shares similarities with bacterial Hsp31, a stress-inducible chaperone[44]. Yet none of this information sheds much light into the pathogenic mechanisms of DJ-1 mutations in PD. Studies of a single DJ-1 mutant, L166P, have shown that it has impaired folding properties and cannot form a homodimer[45,46]. Thus, as a misfolded and less stable protein, DJ-1^{L166P} may cause cytotoxicity by overwhelming the cellular protein degradation systems and by undergoing abnormal subcellular localization, for instance in mitochondria[45]. It remains to be established how accurate this pathogenic scenario is and whether DJ-1^{L166P} reliably reflects the molecular abnormalities of the other known DJ-1 mutations. Furthermore, DJ-1 chiefly localizes in brain glial cells[47], suggesting that neuronal death mediated by DJ-1 mutations may arise from a NON-CELL-AUTONOMOUS process.

PINK1, the new kid on the block

AUTOZYGOSITY mapping of a large consanguineous Sicilian family localized the *PARK6* locus, which is linked to an autosomal recessive form of PD, to chromosome 1p35–36 (ref. 48). *PARK6* has since been linked to an early-onset recessive PD in eight additional families from four European countries[49]. Sequencing of candidate genes within the *PARK6* region in affected members from each family identified two homozygous mutations in the gene encoding PTEN-induced putative kinase-1 (*PINK1*, also known as *BRPK*)[50]. PINK1 is a 581-amino-acid protein with a predicted molecular mass of 62.8 kDa, which shows 95% sequence similarity between human and mouse[51,52]. In adult mice, PINK1 is ubiquitously expressed among tissues, with an apparently high expression in the brain[51]. In both human and mouse PINK1, a serine/threonine kinase domain is the sole known functional domain[51,52]. Presumably PINK1 also has a mitochondrial targeting motif, consistent with the observation that it localizes to the mitochondria in transfected cell lines[50]. If confirmed, this finding may be significant, as a defect in mitochondrial function has been implicated in the pathogenesis of PD[1].

Of the two identified mutations, one is a missense mutation (G309D) in the putative kinase domain and the second is a nonsense mutation (W437OPA) truncating the last 145 amino acids of the kinase domain C terminus[50]. Both mutations are expected to impair PINK1 kinase activity or substrate recognition and to cause PD

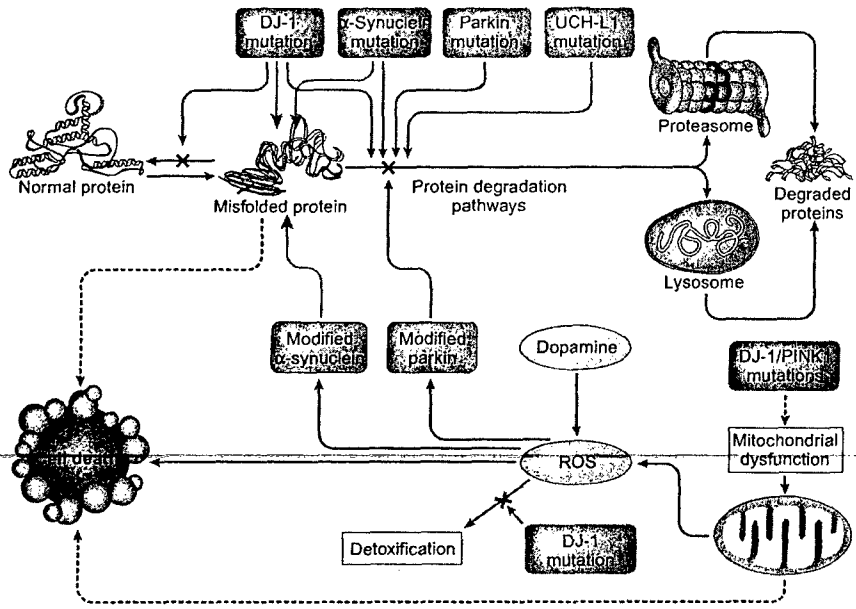

Figure 1 Genetic mutations and the pathogenesis of PD. Misfolded proteins may contribute to PD neurodegeneration. Mutant α-synuclein and DJ-1 may be misfolded (blue arrows), thus overloading the ubiquitin (proteasomal) and lysosomal degradation pathways. Other mutant proteins, such as parkin and UCH-L1, may lack their wild-type function. Both of these proteins, which belong to the ubiquitin-proteasome system, upon mutation may no longer exert their ubiquitin ligase activity, thus damaging the ability of the cellular machinery to detect and degrade misfolded proteins (red arrows). Mutations in DJ-1 may also alter its supposed chaperone activity, disrupting the refolding of damaged proteins or the targeting and delivery of damaged proteins for degradation (red arrows). These different alterations may lead to the accumulation of unwanted proteins, which, by unknown mechanisms (dashed arrows), may lead to neurodegeneration. Oxidative stress generated by mitochondrial dysfunction and dopamine metabolism may also promote protein misfolding as a result of post-translational modifications, especially of α-synuclein and parkin. Oxidative stress in PD may also originate from a defect in the reduced capacity of DJ-1 to detoxify reactive oxygen species, whereas the mitochondrial dysfunction may, at least in part, derive from defective activity and mislocation of DJ-1 and PINK1. Mitochondrial dysfunction, oxidative stress and protein mishandling are thus tightly interconnected in this hypothesized pathogenic cascade. Additional possible interactions have been omitted for clarity.

GLOSSARY

RING domain One of a class of protein domains that consist of two loops that are held together at their base by cysteine and histidine residues that complex two zinc ions. Proteins containing domains of this type are known as RING-finger proteins.

Sumoylation The attachment of SUMO, a ubiquitin-like modifier protein. But in contrast to ubiquitin, which targets proteins for degradation, SUMO seems to affect the subcellular localization of proteins and enhance their stability.

Non-cell-autonomous A genetic trait in which genotypically mutant cells cause other cells (regardless of their genotype) to show a mutant phenotype. By contrast, a cell-autonomous trait is one in which only genotypically mutant cells show the mutant phenotype.

Autozygosity Homozygosity by virtue of parental descent from a common ancestor.

through a loss of function. Yet reduced striatal [^{18}F]dopa uptake revealed by positron emission tomography has been found in asymptomatic *PARK6* heterozygotes, raising the possibility that *PINK1* mutations may operate through haploinsufficiency or a dominant-negative effect[53]. In either case, impaired phosphorylation of PINK1's substrate, especially in mitochondria, is a likely scenario for the pathogenic mechanism of the two mutations.

Although the crucial substrates of PINK1 have yet to be identified, we already know that neuroblastoma cells transiently transfected with either wild-type or mutant PINK1 do not show detectable alterations in viability[50]. In contrast, when these cells are challenged with the proteasome inhibitor MG132, overexpression of wild-type PINK1 mitigates cell death, whereas overexpression of mutant PINK1 neither attenuates nor enhances MG132-mediated cytotoxicity[50]. These results suggest that the loss of PINK1 function renders dopaminergic neurons more vulnerable to injury. This possibility does fit neatly into the concept that interactions between genetic and environmental factors may be responsible for the neurodegeneration in sporadic PD[1].

Conclusion

The shared phenotype associated with the different genetic mutations we have discussed raises the tantalizing possibility of a molecular intersection in the pathogenic mechanisms driven by these distinct PD-causing mutations (Fig. 1). Among various plausible mechanistic hypotheses, available data favor impaired protein degradation and accumulation of misfolded proteins as the unifying factor linking genetic alterations to dopaminergic neurodegeneration in familial PD (Fig. 1). According to this reasoning, α-synuclein and DJ-1 mutations would cause abnormal protein conformations, overwhelming the main cellular protein degradation systems—the proteasomal and lysosomal pathways—whereas parkin and UCH-L1 mutations would undermine the cell's ability to detect and degrade misfolded proteins. The common end result of these different perturbations is thus expected to be a cellular buildup of unwanted proteins that should have been cleared. Minimal defects in this protein turnover machinery may suffice to cause a slow demise of dopaminergic neurons, which may explain the relentless, progressive nature of the disease. This scenario does not, however, explain why an accumulation of misfolded proteins, which is likely to occur in all cells, would inflict greater damage on dopaminergic neurons in familial PD. Perhaps nigrostriatal dopaminergic neurons are less able to cope with 'misfolded protein stress' because of a higher basal load of damaged proteins that is due to dopamine-mediated oxidative events. Also poorly addressed by the above scenario is the link between previously identified factors in PD neurodegeneration, such as mitochondrial dysfunction or oxidative stress, and the molecular events engendered by the PD-causing mutations. The hypothesized mitochondrial location of DJ-1 and PINK1 and the role of DJ-1 in oxidative stress may emerge as crucial in efforts to reconcile the different aspects of the unified pathogenic cascade of PD.

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The authors declare that they have no competing financial interest.

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Therapeutic immunization protects dopaminergic neurons in a mouse model of Parkinson's disease

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Degeneration of the nigrostriatal dopaminergic pathway, the hallmark of Parkinson's disease, can be recapitulated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Herein, we demonstrate that adoptive transfer of copolymer-1 immune cells to MPTP recipient mice leads to T cell accumulation within the substantia nigra pars compacta, suppression of microglial activation, and increased local expression of astrocyte-associated glial cell line-derived neurotrophic factor. This immunization strategy resulted in significant protection of nigrostriatal neurons against MPTP-induced neurodegeneration that was abrogated by depletion of donor T cells. Such vaccine treatment strategies may provide benefit for Parkinson's disease.

Parkinson's disease (PD) is a common neurodegenerative disease characterized clinically by resting tremor, rigidity, slowness of voluntary movement, and postural instability (1). Loss of dopaminergic neurons within the substantia nigra pars compacta (SNpc), intraneuronal cytoplasmic inclusions or "Lewy bodies," gliosis, and striatal dopamine depletion are principal neuropathological findings. With the exception of inherited cases linked to specific gene defects that account for <10% of cases, PD is a sporadic condition of unknown cause (2).

Inflammation increases the risk of PD (3). Experimental disease models show that innate immunity, especially glial inflammatory factors such as proinflammatory cytokines and reactive oxygen and nitrogen species contribute to the degeneration of the nigrostriatal dopaminergic pathway (4). Although less studied than innate immunity, T lymphocytes present in brain tissue may also affect disease progression (5, 6). For example, T cells perform surveillance functions in the nervous system (7, 8), and T cell-deficient mice show enhanced neuronal loss after CNS damage (9, 10). Adaptive immunity, after vaccination with CNS antigens expressed at the lesion site, can attenuate neuronal death. For instance, in optic nerve and spinal cord injuries, encephalitic T lymphocytes directed against myelin-associated antigens positively affect neurodegenerative processes (11–14). Such self-antigen-stimulated T cells may retard neuronal injury by producing neurotrophins (15, 16) or by influencing their production by local glial cells (17).

Based on these prior studies, we theorized that immunization strategies could induce T cells to enter inflamed nigrostriatal tissue, attenuate innate glial immunity, and increase local neurotrophic factor production. To investigate this notion, copolymer-1 (Cop-1; Copaxone, glatiramer acetate), a random amino acid polymer that generates nonencephalitic T cells, which cross-react with myelin basic protein (MBP) in humans (18) and mice (19), was tested in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-intoxicated mice. Cop-1 immunization protects against secondary CNS injury without the encephalitis associated with MBP immunization (20, 21). Moreover, s.c. Cop-1 immunization preferentially incites T cells with a T_H2 phenotype, which secrete antiinflammatory cytokines such as IL-4, IL-10, and transforming growth factor-β (22). We now demonstrate that Cop-1 immune cells administered to MPTP-intoxicated mice by adoptive transfer enter inflamed brain regions, suppress microglial responses, and increase expression of glial cell line-derived neurotrophic factor (GDNF).‡‡ The process was T cell-dependent and led to significant dopaminergic neuronal protection. Because no currently clinically approved therapy prevents progressive degeneration of dopaminergic neurons in PD, we suggest that such a vaccination strategy could be of therapeutic benefit.

Materials and Methods

Animals and MPTP Treatment. Male SJL mice (6–10 weeks old, The Jackson Laboratory) received four i.p. injections at 2-h intervals of either vehicle (PBS, 10 ml/kg) or MPTP-HCl (18 mg/kg of free base in PBS; Sigma). Twelve hours after the last MPTP injection, random mice received adoptive transfers of splenocytes from Cop-1- or ovalbumin (OVA)-immunized mice or no splenocytes (n = 5–9 mice per group per time point). On days 2 and 7 after MPTP intoxication, mice were killed and brains were processed for subsequent analyses. All animal procedures were in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. MPTP handling and safety measures were in accordance with published guidelines (23).

Immunization and Adoptive Transfers. Mice were immunized with a total dose of 200 μg of either Cop-1 or OVA emulsified in complete Freund's adjuvant containing 1 mg/ml *Mycobacterium tuberculosis* (Sigma). Five days after immunization, mice were killed and single-cell suspensions were prepared from the draining inguinal lymph nodes and spleen. MPTP-intoxicated mice received an i.v. injection of 5×10^7 splenocytes in 0.25 ml of Hanks' balanced salt solution. In all adoptive transfer experiments, pooled immunized donor cells were tested for proliferation by [^{3}H]thymidine uptake and/or cytokine expression by ELISA after exposure to immunizing or nonrelevant antigen.

Cytokine Measurements. Donor splenocytes were plated at a density of 1×10^6 cells per ml of tissue culture media [RPMI medium 1640 supplemented with 10% FBS/2 mM L-glutamine/25 mM Hepes/1 mM sodium pyruvate/1× nonessential amino acids/55 μM 2-mercaptoethanol/100 units/ml penicillin/100 μg/ml streptomycin (Mediatech, Herndon, VA)] and stimulated with immunizing antigens. After incubation (37°C at 48 h), supernatants were assayed for IL-10 by ELISA (R&D Systems).

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Abbreviations: PD, Parkinson's disease; SNpc, substantia nigra pars compacta; Cop-1, copolymer-1; MBP, myelin basic protein; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; GDNF, glial cell line-derived neurotrophic factor; OVA, ovalbumin; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein.

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CD90 T Cell Depletion and Flow Cytometry. Donor splenocyte cell suspensions from Cop-1-immunized donors were depleted of T cells using anti-CD90 magnetic beads and magnetic LD columns (Miltenyi Biotec, Auburn, CA). Negatively selected cells ($CD90^-$) were pooled ahead of time and were analyzed for cell purity with a FACSCalibur flow cytometer interfaced with CELLQUEST software (BD Biosciences, Immunocytometry Systems, San Jose, CA) before adoptive transfers. Unfractionated and T cell-depleted populations were stained for T cells using FITC-conjugated anti-CD3 (clone 145-2C11, BD Biosciences, Pharmingen, San Diego) and B cells with phycoerythrin-conjugated anti-B220 (clone RA3-6B2, BD Biosciences, Pharmingen).

Immunohistochemistry and Quantitative Morphology. Seven days after MPTP intoxication, mice were killed and their brains were processed for tyrosine hydroxylase (TH) and thionin staining (24). Total numbers of TH- and Nissl-stained neurons in SNpc were counted stereologically with STEREO INVESTIGATOR software (MicroBrightfield, Williston, VT) by using an optical fractionator (25). Quantitation of striatal TH immunostaining was performed as described (24). Optical density measurements were obtained by digital image analysis (Scion, Frederick, MD). Striatal TH optical density reflected dopaminergic fiber innervation.

Additional primary antibodies used in these studies included rat Mac-1 (1:1,000; Serotec), rabbit glial fibrillary acidic protein (GFAP; 1:1000, DAKO), and rat CD3 (1:800; Pharmingen). Immunostaining was visualized by using diaminobenzidine as the chromogen. For immunofluorescence staining on fresh frozen sections, rabbit anti-CD3 (1:200, DAKO) was used with rat-anti-Mac-1 and goat anti-GDNF (1:100, R & D Systems). Confocal images were obtained with a Zeiss confocal LSM410 microscope.

Cell Tracking. Splenocytes from Cop-1-immunized donors were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFDA SE) by using the Vybrant CFDA SE cell tracer kit (Molecular Probes). Splenocytes (5×10^7) were adoptively transferred into PBS- or MPTP-treated mice. At 2, 8, and 20 h ($n = 3$ mice per time point) after adoptive transfers, mice were killed, their brains were fixed (4% paraformadehyde), and cryostat-cut sections were analyzed by fluorescence microscopy.

RNA Isolation and Real-Time RT-PCR. Total RNA from ventral midbrain and cerebellum ($n = 5$–7 mice per group) was extracted with TRIzol (Invitrogen). RNA was reverse-transcribed with random hexamers and real-time quantitative PCR was performed on cDNA by using the Applied Biosystems PRISM 7000 sequence detector with SYBR green I as the detection system. The murine primer sequences included: Mac-1, 5′-GCCAATGCAACAGGTGCATAT-3′ (forward) and 5′-CACACATCGGTGGCTGGTAG-3′ (reverse); GDNF, 5′-TGTTCTGCCTGGGTGTTGCT-3′ (forward) and 5′-TTGGAGTCACTGGTCAGCG-3′ (reverse). Primers for GAPDH were purchased from Applied Biosystems. Data are presented as a ratio of mean threshold (C_t) target gene expression and GAPDH. Differences between means were analyzed by using one-way ANOVA followed by the least significant difference posthoc test for pairwise comparisons.

Mac-1$^+$ Immunohistochemistry. Midbrain sections (30 μm) from two mice per treatment group (four to six sections per animal) were immunostained for Mac-1. Cell counts were obtained of amoeboid Mac-1$^+$ cells within the SN by using criteria reported (26) and cells per mm^2 was calculated. Numbers of Mac-1-positive cells were averaged for each animal and the mean cells per mm^2 per animal was estimated. The average countable area between treatment groups ranged from 1.92 mm^2 to 2.22 mm^3, and no significant differences in the size of countable areas were observed by ANOVA ($P = 0.063$, $n = 84$ countable areas).

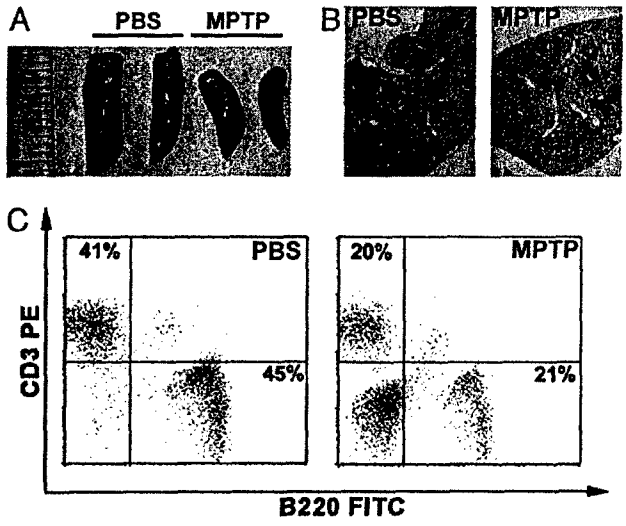

Fig. 1. MPTP-induced immunotoxicity. (*A* and *B*) Seven days after MPTP intoxication, spleen size (*A*) and $CD3^+$ T lymphocyte numbers (*B*) were reduced in spleens of MPTP-treated mice. (*C*) Flow cytometric analysis of splenocytes from PBS (*Left*) and MPTP (*Right*) 2 days after intoxication.

Western Blot Assays. Ventral midbrain protein extracts (25 μg per lane) were fractionated on SDS/4–20% PAGE (Invitrogen), and were then transferred onto PVDF membranes. Membranes were probed with horseradish peroxidase-conjugated anti-mouse IgG or rabbit anti-GFAP (1:15,000; DAKO). Secondary anti-rabbit antibodies conjugated with horseradish peroxidase were visualized by using SuperSignal West Pico chemiluminescent substrate and CCL-XPosure film (Pierce). Immunoblots were stripped and reprobed with antibodies to α-actin (Chemicon) as an internal control.

Measurement of Striatal Catecholamines. Striatal dopamine and its metabolites, dihydroxyphenylacetic acid, and homovanillic acid, were analyzed 7 days after MPTP treatment by reverse-phase HPLC with electrochemical detection (25).

Statistical Analysis. All values are expressed as mean ± SEM. Differences among means were analyzed by one-way ANOVA followed by Bonferroni post hoc testing for pairwise comparison unless otherwise stated. The null hypothesis was rejected at the level of 0.05.

Results

Cop-1 Immunity Confers Dopaminergic Neuroprotection. To test whether Cop-1 immunity confers dopaminergic neuroprotection, MPTP-intoxicated SJL mice received, by adoptive transfer, 12 h after MPTP treatment, 5×10^7 donor splenocytes from nonintoxicated mice previously immunized with either Cop-1 or chicken egg OVA. Replicate MPTP- and PBS-treated mice that did not receive splenocytes served as controls. Adoptive transfer of Cop-1 immune cells to MPTP-treated recipients was used because immunotoxicity precluded active immunization studies. Indeed, MPTP induced significant changes in spleen size with diminished numbers of $CD3^+$ T cells 7 days after MPTP intoxication (Fig. 1 *A* and *B*). Flow cytometric analysis of splenocyte populations revealed a 51% and 53% decrease in $CD3^+$ T cell and $B220^+$ B cell numbers, respectively (Fig. 1*C*). Because MPTP intoxication occurs rapidly and its metabolism into the active toxin, 1-methyl-4-phenylpyridinium (MPP^+), is complete within minutes (27) and is undetectable after 8 h (28), the timing of splenocyte adoptive transfers was designed to avoid confounding effects of MPTP metabolism and its induced hematopoietic toxicity (29). Seven days after MPTP treatment, after which no further dopaminergic neurodegeneration is detected

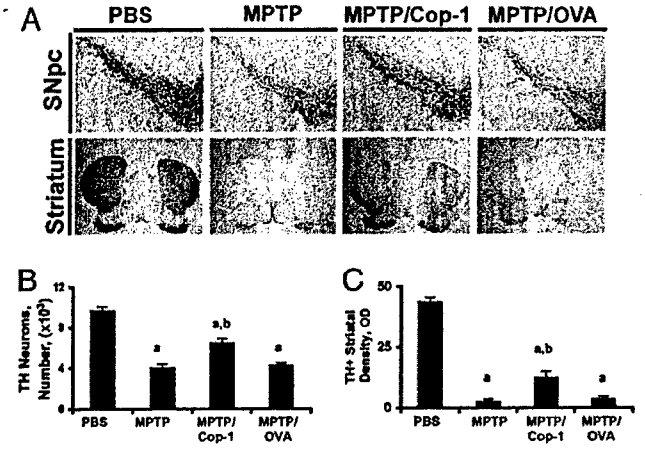

Fig. 2. Cop-1 immunization protects against MPTP-induced dopaminergic neuronal loss. (*A*) Photomicrographs of SNpc and striatum TH immunostaining from PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. (*B*) SNpc TH$^+$ neuronal counts of SNpc TH$^+$ neurons. (*C*) Optical densities of striatal TH$^+$ fibers. Values represent means ± SEM for five to nine mice per group. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b).

(30), mice were transcardially perfused with saline followed by 4% paraformaldehyde, their brains were removed, were cryosectioned, and immunostained for expression of TH, the rate-limiting enzyme in dopamine synthesis (Fig. 2*A*). Stereological counts revealed that MPTP caused a 58% loss of SNpc TH-positive neurons compared with PBS controls (Fig. 2*B*). Similar results were observed in MPTP-injected mice that received splenocytes from OVA-immune donors (MPTP/OVA; Fig. 2 *A* and *B*). In contrast, MPTP-injected mice that received Cop-1 splenocytes (MPTP/Cop-1) exhibited a much smaller reduction in the number of SNpc dopaminergic neurons compared with MPTP or MPTP/OVA animals (Fig. 2 *A* and *B*). Counts of SNpc neurons after Nissl staining with thionin correlated with TH-positive neuron counts ($r = 0.993, P < 0.0001$). This finding confirmed that differences in TH-positive neuron counts were due to numbers of structurally intact neurons and eliminated the possibility that differences resulted from the down regulation of TH itself (Table 2, which is published as supporting information on the PNAS web site, and ref. 30).

Sparing of SNpc dopaminergic cell bodies does not always correlate with protection of their corresponding striatal nerve fibers (25), which is essential for maintaining dopaminergic neurotransmission. To determine whether adoptive transfer of Cop-1 splenocytes affected the integrity of striatal dopaminergic fibers, the density of TH-immunoreactivity in striata (Fig. 2 *A* and *C*) was assessed. MPTP reduced striatal TH density by 94% (MPTP) and 92% (MPTP/OVA) compared with PBS controls (Fig. 2*C*). In contrast, loss of striatal TH density in MPTP/Cop-1 mice (72% loss) was significantly less compared with what was observed in MPTP and MPTP/OVA animals (Fig. 2*C*). The dopaminergic nerve terminals are consistently more affected than the cell bodies in both PD and its MPTP model and are often less amenable to neuroprotection (25, 31). Thus, given the severity of damage at level of the nerve terminals, any significant protection is deemed relevant. Taken together, these findings indicate that Cop-1 immune cells mitigate the deleterious action of MPTP on dopaminergic nerve fibers in the striatum and cell bodies in the SNpc. The ability of splenocytes from Cop-1-immunized mice to confer neuroprotection to myelinated axons is consistent with prior studies where Cop-1 immunization protected against traumatic nerve injury (20).

To determine whether adoptive transfer of Cop-1 immune cells also protects against biochemical deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in striata 7 days after MPTP treatment. Characteristic diminution in striatal dopamine levels by 51% for MPTP-treated mice and 41% for the MPTP/OVA group was observed compared with levels in striata of PBS controls. In contrast, animals that received Cop-1 splenocytes showed only a 4% decrease in striatal dopamine (Table 1). Together, these results indicate that spleen cells from Cop-1-immunized mice protect neuronal dopamine metabolism as well as structural neuronal elements and its projections.

Table 1. Striatal neurotransmitter levels from mice 7 days after MPTP treatment

Treatment	Neurotransmitter levels, ng/mg tissue		
	Dopamine	DOPAC	HVA
PBS ($n = 4$)	10.0 ± 0.1	0.9 ± 0.2	8.8 ± 0.8
MPTP ($n = 6$)	4.9 ± 0.05*	1.4 ± 0.2	5.4 ± 1.1*
COP-1/MPTP ($n = 5$)	9.6 ± 1.1	1.3 ± 0.2	7.7 ± 1.0
OVA/MPTP ($n = 6$)	6.0 ± 0.07*	2.3 ± 0.2†	5.0 ± 0.2*

Values in parentheses are the mean ± SEM for no. of mice per treatment group. DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid.
*$P < 0.01$ compared with PBS and COP-1.
†$P < 0.02$ compared with all groups.

Cop-1 Immune Cells Reduce Microglial Reactions. Based on studies that demonstrate antiinflammatory cytokine profiles by Cop-1-reactive T cells (19, 22), we theorized that the protective effects of Cop-1 immune cells resulted from the modulation of glial inflammatory responses. In line with previously reported results, our immunization strategy generated T cells that proliferate (data not shown) and secrete IL-10 and IL-4 in response to MBP and/or Cop-1 (Fig. 3 *A* and *B*). Because the active phase of neuronal death and neuroinflammatory activities peak at ≈2 days after MPTP injection (25, 30), we assessed lymphocyte infiltration and IgG in the nigrostriatal region at this time point. CD3$^+$ T cells were detected within nigrostriatal tissue (Fig. 3 *C* and *D*) in all mice after MPTP intoxication and adoptive transfer. However, differences were not observed in the ventral midbrain IgG by either Western blot (Fig. 3*E*) or immunohistochemical tests (data not shown). These findings suggested that T cells, not IgG, play the principal roles in the neuroprotective activities observed in these studies. To confirm whether infiltrating T cells in immunized mice were donor-derived, splenocytes were labeled *ex vivo* with the succinimidyl ester of carboxyfluorescein diacetate (Molecular Probes) and transferred intravenously to MPTP mice. As early as 2 h after adoptive transfer and for 20 h thereafter, carboxyfluorescein diacetate-labeled lymphocytes were readily observed both in ventral midbrains and striata of MPTP mice. No labeled cells were found in the cerebellum, a region not afflicted by MPTP. These data demonstrate that donor-derived T cells rapidly enter affected regions of the brain during active inflammation and neuronal loss.

Based on observations that peripheral lymphocytes enter and accumulate in areas of tissue damage early after cell transfer and at times of peak inflammation, we assessed the potential of Cop-1 immune cells to regulate MPTP-induced microglial reactions. In MPTP-treated animals, CD3$^+$ T cells were readily seen in close association with activated microglial cells (Fig. 3*F*); the latter evidenced by increased expression of Mac-1 (CD11b), a cell-surface receptor for complement that is up-regulated by activated microglia in both PD and the MPTP model (31). The evidence that Cop-1 immune spleen cells secreted IL-10 and IL-4 upon *in vitro* stimulation with Cop-1 or MBP (Fig. 3 *A* and *B*), suggested that T cell cytokines may affect glial cell function. Because MPTP-induced neurodegeneration may be attenuated by microglia deactivation (25, 31, 32), we analyzed the ventral midbrain for Mac-1 gene expression by real-time RT-PCR 24 h after adoptive transfer of

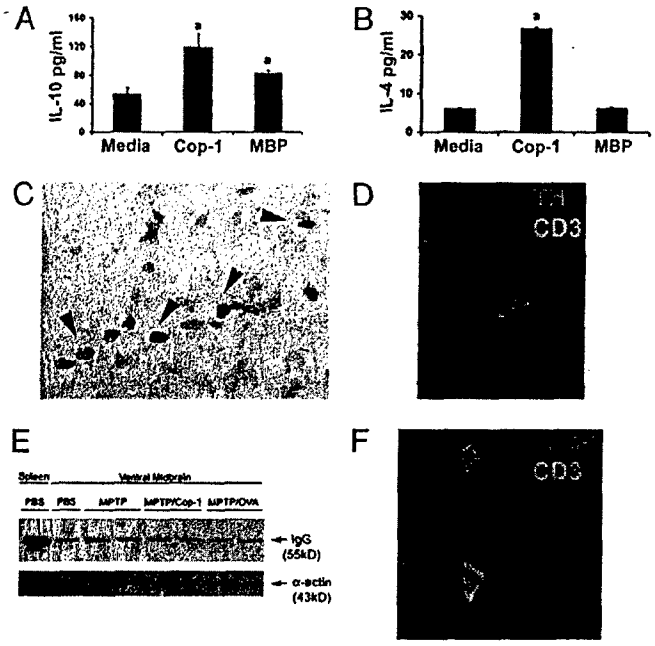

Fig. 3. Cytokine secretion by Cop-1 donor immune cells and T cell infiltration of the SNpc. (*A* and *B*) IL-10 (*A*) and IL-4 (*B*) secretion by Cop-1-immunized splenocytes cultured in media or stimulated with Cop-1 or MBP (30 μg/ml). Values are means of IL-10 or IL-4 concentrations ± SEM for three to four mice. a, $P < 0.05$ compared with PBS treatment group. (*C*) $CD3^+$ T cells in the SNpc of MPTP-intoxicated mice 2 days after adoptive transfer of Cop-1 splenocytes (arrows). (*D*) $CD3^+$ T cells in proximity (green) to TH^+ neurons (red) within the SNpc of an MPTP mouse. (*E*) Western blot analysis for IgG in ventral midbrains after adoptive transfer of splenocytes. (*F*) Ventral midbrain $CD3^+$ T cells (green) in direct contact with Mac-1^+ cells (red; magnification: ×2,000).

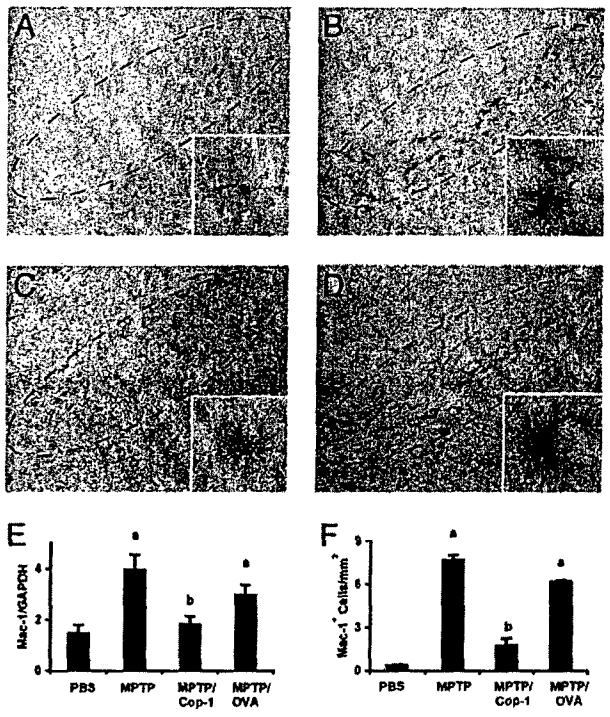

Fig. 4. Cop-1 immunization reduces MPTP-induced microglial reaction in the SNpc. (*A–D*) Mac-1 immunostaining within the SNpc (area circumscribed by dashed line and *Insets* at ×100 magnification) from PBS (*A*), MPTP (*B*), MPTP/Cop-1 (*C*), or MPTP/OVA (*D*) groups. (*E*) Real-time RT-PCR assessment of Mac-1/GAPDH mRNA from ventral midbrain. (*F*) Counts of Mac-1^+-reactive microglia from SNpc. $P < 0.05$ compared with PBS (a), MPTP (b), and MPTP/OVA (b) groups.

Cop-1 splenocytes (48 h after last MPTP injection). In agreement with prior studies (31), brains from MPTP-treated animals showed significant increases in Mac-1 mRNA. In contrast, MPTP/Cop-1 mice showed lower Mac-1 expression compared with both MPTP and MPTP/OVA animal groups (Fig. 4*E*). Immunohistochemical staining for cell-surface expression of Mac-1 in the ventral midbrain 48 h after adoptive transfer reflects levels of Mac-1 mRNA (Fig. 4 *A–D*). In PBS control mice, Mac-1 expression was associated with small microglial cells having thin ramifications (Fig. 4*A*). MPTP-injected and MPTP/OVA mice showed intense Mac-1 immunoreactivity, which revealed larger microglial cells with thicker short ramifications (Fig. 4 *B* and *D*). In MPTP/Cop-1 mice, Mac-1^+ cells were smaller, with finer processes approximating those in PBS controls (Fig. 4*C*). Enumeration within the SN of Mac-1^+ microglia with an activated phenotype showed a significant reduction in reactive microglia in the MPTP/Cop-1 group compared with MPTP- or MPTP/OVA-treated mice (Fig. 4*F*). Correlation analysis of Mac-1 mRNA expression and Mac-1^+ microglia counts from PBS-, MPTP-, MPTP/Cop-1-, and MPTP/OVA-treated groups indicated a strong correlation ($r = 0.76$, $P = 0.03$). Taken together, these data indicate that Cop-1 splenocytes are capable of attenuating MPTP-induced microglial reactions.

Although Cop-1 immune transfer significantly diminished the microglial reaction, astrocyte morphology was not affected. Expression of the astrocyte-specific antigen, GFAP, was comparable among all MPTP treatment groups as revealed by Western blot analysis of ventral midbrain 2 days after MPTP administration (data not shown). Astrocytosis by day 7 after MPTP treatment, shown by enhanced GFAP immunostaining and astrocyte morphology was similar among MPTP-treated groups, irrespective of passive immunization strategies (data not shown).

Neuroprotection Is T Cell-Dependent. As stated, T cells entered the damaged nigrostriatal tissue after MPTP intoxication in the absence of any noticeable alterations in nigrostriatal IgG levels. This finding suggested that the cellular arm of the immune system was responsible for neuroprotection. To test this hypothesis, T cell-depleted splenocytes from Cop-1-immunized mice were prepared by anti-CD90-conjugated magnetic beads. This action resulted in the removal of >90% of $CD3^+$ T lymphocytes without affecting B cell ($B220^+$) populations (Fig. 5*A*). In the experiments, MPTP-treated mice received unfractionated or T cell-depleted splenocytes from Cop-1-immunized donors, unfractionated splenocytes from OVA-immunized donors, or no splenocytes. On day 7, mice were killed and brain tissue was immunostained for TH content in the SNpc and striatum (Fig. 7, which is published as supporting information on the PNAS web site). A significant reduction in the number of TH-positive neurons within the SNpc was observed in MPTP-treated mice that received no splenocytes or splenocytes from OVA-immunized donors (Fig. 5*B*). Significant neuroprotection was afforded to MPTP-treated recipients of splenocytes from Cop-1-immunized mice (Fig. 5*B*). However, neuroprotection was ablated in mice that received T cell-depleted Cop-1 splenocytes (Fig. 5*B*). Parallel changes in striatal dopaminergic nerve fibers was also demonstrated. The diminution of TH optical density in striatal sections was significantly less in MPTP-treated recipients of unfractionated Cop-1 splenocytes compared with MPTP-treated control groups; however, this neuroprotection was ablated in recipients of T cell-depleted Cop-1 splenocytes (Fig. 5*C*). These results indicate that T cells from Cop-1-immunized donors are required for the observed neuroprotective activities.

Cop-1 Immunization Increases Expression of GDNF in Ventral Midbrain. Finally, we investigated whether Cop-1 immunization affects neurotrophin production at the site of disease. GDNF mitigates neu-

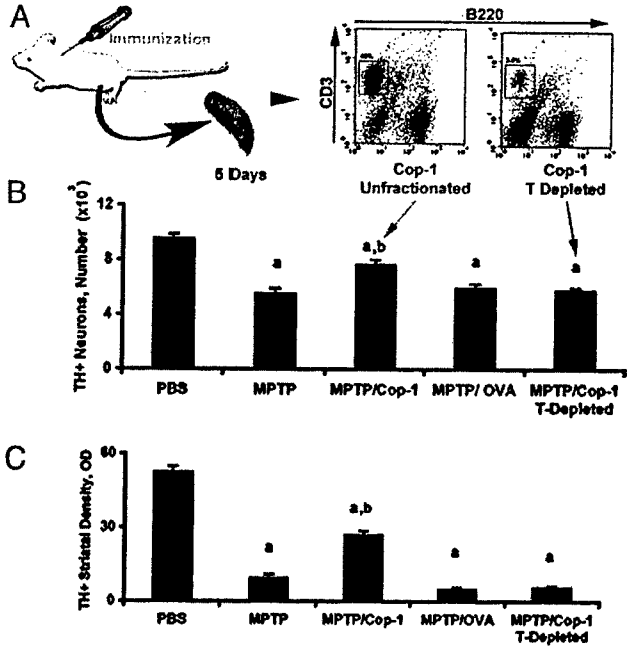

Fig. 5. T cell depletion ablates Cop-1-mediated dopaminergic neuroprotection. (*A*) Flow cytometric analysis of Cop-1 immune splenocytes before (unfractionated) and after T cell depletion. (*B*) Counts of SNpc TH^+ neurons for PBS (n = 5), MPTP (n = 7), MPTP/Cop-1 (n = 8), MPTP/OVA (n = 8), and MPTP/Cop-1/T cell-depleted groups (n = 6). (*C*) Densities of striatal TH^+ fibers. Values are means ± SEM. $P < 0.01$ compared with PBS (a), MPTP (b), and MPTP/OVA (b), MPTP/Cop-1/T (b)-depleted groups.

rodegenerative processes in MPTP animals and leads to symptomatic recovery after dopaminergic injury (33), This finding formed the basis for PD clinical trials that so far have yielded promising results (34). In our study, we quantitated by real-time RT-PCR analysis, GDNF mRNA levels in ventral midbrains from PBS, MPTP, MPTP/Cop-1, and MPTP/OVA mice 20 h after adoptive transfer. MPTP/Cop-1 mice showed significantly greater levels of ventral midbrain GDNF mRNA compared with all other groups (Fig. 6*A*). To identify the cellular source of GDNF within the SN

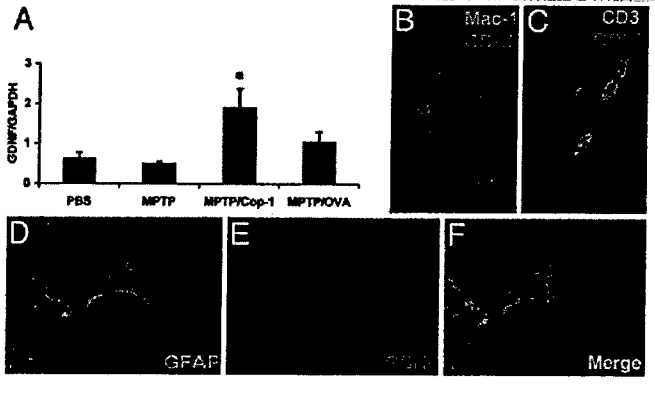

Fig. 6. GDNF expression in MPTP-intoxicated mice after adoptive transfer of Cop-1 splenocytes. (*A*) Real-time RT-PCR of GDNF mRNA expression from ventral midbrains of PBS, MPTP, MPTP/Cop-1, or MPTP/OVA groups. Values represent ratios of GDNF mRNA normalized to GAPDH and are means ± SEM for five to six mice per group. $P < 0.05$ compared with PBS (a), MPTP, and MPTP/OVA groups. (*B–F*) Confocal microscopy of SNpc from MPTP-treated recipients of splenocytes from Cop-1-immunized mice showing GDNF immunostaining (red) (*B–D* and *F*) and $Mac\text{-}1^+$ (green) microglia (*B*), $CD3^+$ (green) T cells (*C*), and $GFAP^+$ (green) astrocytes (*D* and *F*). Magnification: ×2000.

of MPTP/Cop-1 mice, sections were double immunostained for GDNF and cell markers. Analysis by confocal microscopy demonstrated that GDNF expression colocalized with cells expressing GFAP, but not with CD3 or Mac-1 (Fig. 6 *B–F*). These data suggest that astrocytes, not T cells or microglia, are the primary source of GDNF production in this model.

Discussion

Epidemiological, immunopathological, and animal model studies support the notion that innate immunity affects nigrostriatal dopaminergic neurodegeneration in PD (2, 35). Many of the pathogenic processes operative in PD are recapitulated in MPTP-intoxicated animals. For example, animals injected with MPTP exhibit early microglial-associated neuroinflammatory events and subsequent nigrostriatal degeneration. Based on a number of prior studies linking neuroinflammation to neurodegenerative processes, we hypothesized that negatively regulating innate immunity in the CNS through T_H2-polarized adaptive immune responses through vaccination could lead to positive disease outcomes. Consistent with this idea, we demonstrate that passive immunization with Cop-1 immune cells into acutely MPTP-intoxicated mice protects the nigrostriatal dopaminergic system. This finding was evidenced by higher numbers of surviving SNpc TH^+ neuronal bodies and striatal fibers, in addition to elevated striatal dopamine levels in MPTP mice receiving Cop-1 immune cells. Taken together, the data indicate that Cop-1 immune cells accumulate specifically in affected brain areas during the most active phase of MPTP-induced neurodegeneration (30), and by so doing, trigger a T cell-dependent neuroprotective response.

The neuroprotection seen in our studies could result as a consequence of T_H1 (proinflammatory, IFN-γ) or a T_H2 or T_H3 (antiinflammatory, IL-10, IL-4, and TGF-β) immune response. However, Cop-1 immunization, in particular, is well known to generate T_H2 or T_H3 T cells (19, 22), which secrete cytokines known to suppress innate immunity (36–38). Cop-1 immunization, in the MPTP model, could exploit immunoregulatory activities of T_H2 or T_H3 T cells and thus provide a vehicle to attenuate microglial neurotoxic responses. Several of our observations support the notion that this scenario may underlie, at least in part, Cop-1 neuroprotective effects in the MPTP model. First, infiltration of the nigrostriatal pathway with donor-derived T cells was seen in close proximity to or in direct cell–cell contact with activated microglia. Second, a marked decrease in MPTP-associated microglial responses was observed after transfer of Cop-1 immune cells. This finding was supported by a profound reduction of ventral midbrain Mac-1 mRNA content and SNpc Mac-1 immunostaining. Third, MPTP-associated astrocytosis, a putative neuroprotective response, remained unchanged by passive immunization with Cop-1 cells. Fourth, IL-10 and IL-4, but not IFN-γ, was secreted by the Cop-1 cells in laboratory assays, providing evidence for the induction of an antiinflammatory T_H2 phenotype in affected brain tissue. Taken together, these results suggest that Cop-1 immune cells in MPTP mice can attenuate the microglial inflammatory responses that contribute to nigrostriatal dopaminergic neurodegeneration.

In addition to targeting the innate immune system, this therapeutic vaccine strategy was shown to augment GDNF within brain regions of active disease. It is likely that this effect is also implicated in the neuroprotective activities of Cop-1 because GDNF delivered to MPTP-intoxicated animals shows significant benefit (33). This observation may also be relevant to human disease given the therapeutic benefits of surgically implanted pumps which directly infuse GDNF into affected dopaminergic structures of PD patients in early human clinical trials (34). Activated T cells express both neurotrophins (39) and the neurotrophic factor receptors, trkB and trkC (40), thus providing sufficient mechanistic means to establish T cell–neuron communications. Consistent with this view, T cells can increase local CNS neurotrophic factor production *in vivo* (11). Our data demonstrate a dramatic increase in ventral midbrain

GDNF expression in Cop-1-immunized MPTP-injected mice. Interestingly, confocal microscopy revealed GDNF in astrocytes, but not in microglia or infiltrating T cells. Thus, these findings suggest that Cop-1 immune cells stimulate the local production of GDNF by astrocytes. In keeping with this idea, T cell cytokines are well known to affect the regulation of neurotrophins (17, 41) that in turn, could actively participate in the observed Cop-1-induced neuroprotective effects.

To our knowledge, this is the first time that a vaccine strategy has been used to confer neuroprotection for dopaminergic neurons. We posit that Cop-1-specific T_H2 cells, which recognize MBP, simultaneously suppress cytotoxic inflammatory responses and increase local neurotrophic factor production. It is possible that both mechanisms converge to ultimately abate the dopaminergic neurodegenerative process that occurs in the MPTP model of PD. In this regard, Cop-1 vaccination reflects the anticipated outcomes of gene therapy. Indeed, both approaches attempt to deliver factors that would attenuate disease to damaged microenvironments. This method is implemented to enhance the therapeutic index by delivering maximal levels of factors to specific diseased areas, thus minimizing system toxicity. Still, immunization avoids the inherent limitation of gene delivery and, by directing immune cells to areas of injury and producing a spectrum of disease mitigating factors, positively alters the neurodegenerative process. Additional studies performed in the MPTP model wherein animals analyzed over time and TH^+ neuronal counts substantiated with other tests, including behavioral and spectroscopic assays, may serve to further validate our experimental observations. In keeping with this concept, preliminary reports from our laboratories based on measures of *N*-acetyl aspartate (a biochemical neuronal marker) by using magnetic resonance spectroscopy and immunopathological coregistration and reverse-phase HPLC in the SNpc confirmed the neuroprotective effects of Cop-1 immune cells in MPTP-injected mice.[§§]

We found unexpectedly that MPTP induces a profound toxicity on cellular components of the peripheral immune system. Adoptive transfer of Cop-1 immune cells to MPTP recipient animals was used as immunotoxicity precluded active immunization. Moreover, our initial work used adoptive transfers with whole splenocyte populations to determine whether collective immune responses elicited against Cop-1 and reflecting active immunization could elicit neuroprotective activities in an animal model of PD. This step is critical in preclinical studies because both T and B cells can affect outcomes in nerve injury models (9) and may work in concert in doing so (42). Indeed, the pharmacokinetics of MPTP are well studied and demonstrate that the toxin is rapidly metabolized in mice and no longer detectable 8 h after the final dose (28). Although passive transfer is commonly performed in humans, there are no contraindications for PD patients to receive direct vaccination with Cop-1 or other related antigens that might elicit similar neuroprotective responses.

We conclude that this report opens a field of investigation toward the development of neuroprotective therapeutic modalities for PD. The reported Cop-1-specific immune-mediated neuroprotection has direct implications for the treatment of PD. As a Food and Drug Administration-approved and well tolerated drug, Cop-1 has been used effectively in patients with chronic neuroinflammatory disease such as relapsing remitting multiple sclerosis for more than a decade. Given the safety record of Cop-1 and that current treatments for PD remain palliative, such a vaccination strategy represents a promising therapeutic avenue that can readily be tested in human clinical trials.

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L-3-Hydroxyacyl-CoA Dehydrogenase II Protects in a Model of Parkinson's Disease

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The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) impairs mitochondrial respiration and damages dopaminergic neurons as seen in Parkinson's disease (PD). Here, we report that L-3-hydroxyacyl-CoA dehydrogenase type II/amyloid binding alcohol dehydrogenase (HADH II/ABAD), a mitochondrial oxidoreductase enzyme involved in neuronal survival, is downregulated in PD patients and in MPTP-intoxicated mice. We also show that transgenic mice with increased expression of human HADH II/ABAD are significantly more resistant to MPTP than their wild-type littermates. This effect appears to be mediated by overexpression of HADH II/ABAD mitigating MPTP-induced impairment of oxidative phosphorylation and ATP production. This study demonstrates that HADH II/ABAD modulates MPTP neurotoxicity and suggests that HADH II/ABAD mimetics may provide protective benefit in the treatment of PD.

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Parkinson's disease (PD) is a common neurodegenerative disease whose main neuropathological feature is the loss of the substantia nigra pars compacta (SNpc) dopaminergic neurons.[1] Although its cause remains unknown, the mechanism of SNpc dopaminergic neuronal death may involve a defect in oxidative phosphorylation.[1] Indeed, reduction in the activity of complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain has been reported in PD tissues (reviewed in Greenamyre and colleagues[2]), especially in the brain[3] and platelets.[4,5] Supporting the significance of this mitochondrial defect in SNpc neuronal death are the observations that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that blocks complex I,[6] reproduces the hallmarks of PD in humans.[7] Furthermore, alternative brain energy substrates, such as the ketone body D-β-hydroxybutyrate or creatine, by improving oxidative phosphorylation, do attenuate dopaminergic neurodegeneration caused by the parkinsonian toxin MPTP.[8–10]

Type II L-3-hydroxyacyl-CoA dehydrogenase/amyloid β peptide binding alcohol dehydrogenase (HADH II/ABAD) is predominantly a mitochondrial enzyme[11–13] that belongs to the short-chain dehydrogenase/reductase superfamily.[14] HADH II/ABAD catalyzes reversibly the third step of fatty acid β oxidation in mitochondria, converting L-3-hydroxyacyl-CoA in the presence of NAD^+ to 3-ketoacyl-CoA, NADH, and H^+.[15] HADH II/ABAD appears to be a multifunctional enzyme with a broad range of substrates.[16] In a murine cerebral ischemia model, HADH II/ABAD overexpression reduces infarct size and neurological deficit scores presumably by enhancing the flux of acetyl-CoA through the tricarboxylic acid (TCA) cycle and by increasing ATP levels in the brain.[17] Although overexpression of HADH II/ABAD is protective in acute brain injury models,[17] its role in a chronic neurodegenerative process, such as in PD, is not known. In this study, we show that not only is HADH II/ABAD downregulated in the SNpc of both PD patients and MPTP-treated mice, but also its overexpression in transgenic mice attenuates MPTP-induced dopaminergic neurodegeneration and ATP depletion. These results indicate that HADH II/ABAD may contribute to determining the fate of compromised SNpc dopaminergic neurons.

Materials and Methods

Animals and Treatment

Characteristics of the transgenic (Tg) mice with approximately four fold increased expression of HADH II/ABAD in neurons can be found in Yan and colleagues.[17] In these an-

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imals, expression of human HADH II/ABAD is driven by the platelet-derived growth factor B-chain promoter. Male C57/bl transgenic HADH II/ABAD hemyzygotes were mated with female wild-type C57/bl mice to yield transgenic and wild-type littermates. Genotyping by polymerase chain reaction was performed on DNA extracted from mouse tails using the following pair of primers: 5′-AGGGCAGAGGAGCGTGTGT-3′ (forward) and 5′-GGCAGCAGCGTGTCGGAGCG-3′ (reverse) and polymerase chain reaction amplification conditions: denaturation at 95°C for 20 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute for 30 cycles. Eight- to 10-week-old mice received one intraperitoneal injection of MPTP-HCl (30mg/kg/day of free base) or saline for 5 consecutive days and were killed at 0, 2, 4, 7, and 21 days after the last injection.

Immunostaining and Quantitative Morphology in Mouse Samples

Twenty-one days after the last MPTP injection, mice were killed and their brains were processed for immunohistochemical studies following our standard protocol,[9] using a polyclonal anti-tyrosine hydroxylase (TH) (1:1,000; Calbiochem, San Diego, CA) or a monoclonal anti-HADH II/ABAD (1:50).[18] Total numbers of SNpc TH-positive neurons were counted using the stereological optical fractionator method.[19] Striatal optical density (OD) of TH fibers[9] was determined using the Scion Image program. Colocalization of HADH II/ABAD with TH was studied using double immunofluorescence followed by confocal microscopic analyses in naive mice.

Quantification of Apoptotic Cells

Four days after MPTP injections, mice were killed and their brains were processed for TH immunostaining as described above and were counterstained with thionin for Nissl substances. Total numbers of apoptotic cells were counted as previously described.[20,21]

Immunoblots

Tissue proteins from ventral midbrains of MPTP- and saline-treated mice and of human brains were isolated[21] and immunoblotted[18] using primary monoclonal antibodies to either HADH II/ABAD (1:15,000), β-actin (1:1,500; Sigma-Aldrich, St. Louis, MO), or polyclonal anti-TH (1:1,000). To assess the translocation of HADH II/ABAD from the matrix to the mitochondrial membrane, brain mitochondria from naive mice were prepared as for the polarographical studies (see below). All mitochondrial preparations had an average respiratory control ratio of ≥5. At the end of the incubation period with different substrates and inhibitors, potassium thiocyanate (150mM final concentration) was added to the mixture to stabilize the bound complex,[22] before they were freeze-thawed five times in liquid nitrogen. The samples were then centrifuged at 150,000*g* 1 hour, and the pellet was resuspended in the assay buffer. Mitochondrial membrane protein was immunoblotted using a primary antibody to either HADH II/ABAD (1:15,000), cytochrome oxidase (COX; subunit IV; 1:1,1000; Molecular Probes, Eugene, OR), or heat shock protein-60 (HSP-60, 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA).

Measurement of Striatal Dopamine and Its Metabolites

Twenty-one days after the last MPTP injection, striatal levels of dopamine and its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were measured by high-performance liquid chromatography with electrochemical detection as described previously.[9]

MPTP Metabolism, Uptake of MPP^+, and Measurement of Lactate Levels

Striatal MPP^+ levels, synaptosomal uptake of $[^3H]$-MPP^+ and mitochondrial accumulation of $[^3H]$-MPP^+ were determined as described previously.[9,23] Lactate production was measured in mouse striatal sections in the presence of MPP^+ as described previously.[23]

Polarography

Oxygen consumption in brain mitochondria was monitored as described[9] using a Clark-type electrode (Hansatech Instruments, PP System, Haverhill, MA). To assess mitochondrial respiration mediated through complex I, we preincubated mitochondria with 10mM glutamate and 5mM malate in the absence or presence of MPP^+ (5 minutes) or rotenone (2.5 minutes) before 500μM ADP was added to induce state 3 respiration. For complex II– (succinate-ubiquinone oxidoreductase) mediated respiration, mitochondria were preincubated for 2.5 minutes with 10mM succinate and 1μM rotenone in the absence or presence of the complex II inhibitor malonate before 500μM ADP was added. For complex IV– (cytochrome oxidase) mediated respiration, mitochondria were preincubated for 2.5 minutes in the absence or presence of the complex IV inhibitor potassium cyanide before 10mM ascorbate and 0.2mM N,N,N′,N′-tetramethyl-benzendiamine (Cayman Chemical, Ann Arbor, MI) were added to induce oxygen consumption.

Mitochondrial ATP and Hydrogen Peroxide Measurements

Samples were prepared under the identical conditions as those for the polarographical studies. ATP was measured using a luciferase luminometric assay as previously described.[9] Hydrogen peroxide, converted from superoxide by manganese-superoxide dismutase, was measured using 5μm Amplex Red and 5U/ml horseradish peroxidase as described previously.[9]

Activities of Mitochondrial Respiratory Chain Complexes in the Mouse Brain

The measurements of activities of mitochondrial complexes I, II, and IV in both brain mitochondria and brain homogenates were performed as previously described[9,24] with several modifications. In brief, freeze-thawed lysed samples (50μg) were used for complex I and II assays. For complex I activity, the oxidation of NADH (EC = $6.23 mM^{-1}cm^{-1}$) by complex I was monitored at 340nm. For complex II activity, the reduction of dichlorophenolindophenol was monitored at 600nm (EC = $19.1 mM^{-1}cm^{-1}$). For complex IV activity, samples (10μg) were added to assay medium (20mM KH_2PO_4, 30mM *n*-dodecyl-β-D-maltoside and 1%

reduced cytochrome c) to initiate the reaction. The oxidation of cytochrome c was measured as the initial rate at 550nm (EC = 19.1mM^{-1}cm^{-1}). Reduced cytochrome c, used as a substrate for complex IV, was prepared fresh by adding a few grains of sodium hydrosulfide as previously described[25] such that the absorbance was in the range of 1.8 to 2.0.

Citrate Synthase Activity

Samples (10μg) were added to the assay medium (0.1M Tris-HCl, pH 8.1, containing 1.0mM 5,5′-dithiobis-[2-nitrobenzoic acid], 10mM acetyl-CoA, and 0.1% Triton X-100) at 30°C. Freshly prepared 10mM oxaloacetate was added to initiate the reaction. The reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) was monitored at 412nm (EC = 13.6mM^{-1}cm^{-1}).

Immunostaining of Human Samples

Human tissue samples were obtained from the Parkinson Brain Bank at Columbia University. Age at death and interval from death to tissue processing were 72.2 ± 8.8 years and 13.0 ± 3.5 hours (mean ± SEM), respectively, for the control group (n = 10) and 77.2 ± 2.3 years and 10.1 ± 2.4 hours, respectively, for the PD group (n = 11). Paraffin-embedded sections (7μm) were deparaffinized and microwaved in 10mM citrate buffer (pH 6.0) for antigen retrieval before incubation with the anti-HADH II/ABAD (1:50). As a negative control, anti–HADH II/ABAD antibody was preabsorbed with excess recombinant HADH II protein (50μg/ml, generated as described in Yan and colleagues[17]). Immunostaining was visualized using 3,3′-diaminobenzidine with cobalt/nickel enhancement.

Statistical Analysis

All values are expressed as mean ± SEM. One-way or two-way analysis of variance was used (unless indicated otherwise) followed by Newman–Keuls post hoc testing for pairwise comparison. The null hypothesis was rejected at the 0.05 level.

Results

HADH II/ABAD Protein Levels Are Reduced in Postmortem Samples from Parkinson's Disease Patients

Although HADH II/ABAD has been shown to be expressed ubiquitously in human brains,[18,26] to assess the relevance of HADH II/ABAD in PD, we asked whether this enzyme is indeed expressed in SNpc dopaminergic neurons, and whether its expression level is altered by the disease process. Using Western blot analyses, we detected a significant reduction in HADH II/ABAD levels in postmortem ventral midbrain samples from PD patients as compared with those from the control subjects (Fig 1A); however, in striatal and cerebellum tissues, HADH II/ABAD levels did not differ between the two groups (data not shown).

Immunohistochemical studies confirmed the ubiquitous expression of HADH II/ABAD in human neurons (not shown), including SNpc dopaminergic neurons, which are readily identifiable by their content of the brown pigment neuromelanin (see Fig 1B–F). Also apparent was a consistently fainter HADH II/ABAD immunoreactivity (blue-gray) in SNpc dopaminergic neurons in PD patients (see Fig 1D, F) compared with the control subjects (see Fig 1C, E). HADH II/ABAD immunostaining also was detected in the core of the prototypical PD proteinacious intraneuronal inclusions, Lewy bodies (see Fig 1D, arrow). These findings indicate that HADH II/ABAD is present in both normal and diseased SNpc dopaminergic neurons and that its expression is reduced in these ventral midbrain neurons from PD patients.

HADH II/ABAD Is Also Reduced in Mouse Ventral Midbrain after MPTP Injection

To assess whether the reduction in HADH II/ABAD seen in PD tissues is not simply a nonspecific alteration due to dying neurons, we turned to the MPTP mouse

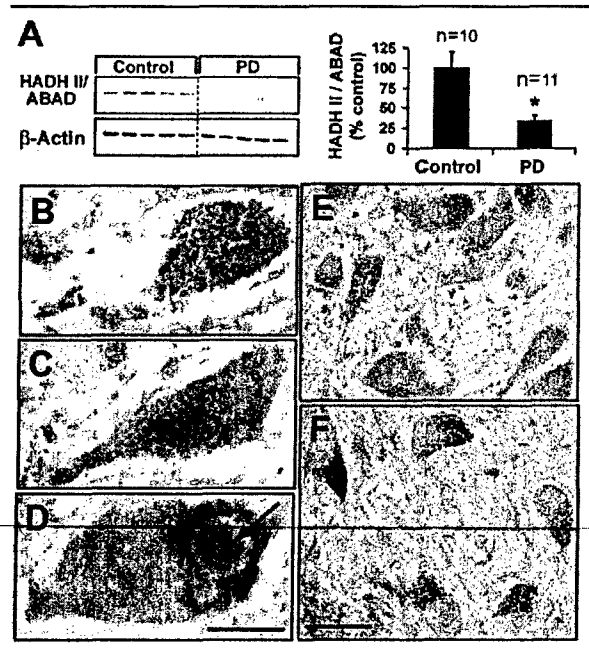

Fig 1. Reduction of HADH II/ABAD levels in Parkinson's disease (PD) patients. Western blot analyses (A) of postmortem ventral midbrain samples show PD patients have significantly lower levels of HADH II/ABAD than control subjects. HADH II/ABAD levels were quantified as the ratio of HADH II/ABAD over β-actin and expressed as percentage of control. Immunohistochemical studies confirm the reduction of this protein in dopaminergic neurons (B–F; brown, neuromelanin; blue-gray, HADH II/ABAD) of PD patients (D, F; arrow *in D: Lewy body) as compared with those of the control subjects (C, E). As a negative control, HADH II/ABAD recombinant protein was added to preabsorb the HADH II/ABAD antibody (B). Data represent mean ± SEM of 10 (control) and 11 (PD) subjects per group,* (asterisk) $p < 0.05$ *compared with control subjects. Scale bars: 25μm (B–D) and 50μm (E, F).*

model of PD. As found in control human samples, mouse ventral midbrain sections immunostained for HADH II/ABAD showed this enzyme is expressed ubiquitously in cells exhibiting essentially a neuronal morphology, including those in the SNpc (Fig 2A–D). By confocal microscopy, HADH II/ABAD immunoreactivity (see Fig 2B) was demonstrated to colocalize (see Fig 2D) with TH (see Fig 2C), a marker for dopaminergic cells. Thus, these results indicate that HADH II/ABAD is highly expressed in mouse neurons including SNpc dopaminergic cells.

To assess ventral midbrain, striatal, and cerebellar levels of HADH II/ABAD during the neurodegenerative process, we performed time course studies in MPTP-injected mice. Like in human samples, ventral midbrain expression of HADH II/ABAD, assessed here by its tissue protein contents (see Fig 2E), was reduced in MPTP-injected mice compared with saline-injected controls. In contrast with HADH II/ABAD, TH levels were not significantly reduced under this MPTP regimen, indicating that the reduction of HADH II/ABAD was not secondary to the loss of TH neurons. The nadir of ventral midbrain HADH II/ABAD protein contents occurred between 2 and 7 days after MPTP injections (see Fig 2E), a period of time that corresponds to the most active phase of apoptosis in SNpc seen with this MPTP regimen.[21] Thereafter, HADH II/ABAD levels progressively increased back to control levels. In contrast with the ventral midbrain region, HADH II/ABAD protein levels did not differ between MPTP- and saline-injected mice in the cerebellum and in the striatum (data not shown).

HADH II/ABAD Protects against MPTP-Induced Neurodegeneration

In light of the MPTP-induced SNpc HADH II/ABAD downregulation, we assessed the role of this enzyme in MPTP neurotoxicity by comparing its toxic effects in transgenic mice overexpressing HADH II/ABAD specifically in neurons and in their wild-type littermates. In saline-injected mice, no difference in dopaminergic neurons was detected between the two groups of animals (Fig 3A, C, I). MPTP caused a marked loss of SNpc TH-positive neurons in wild-type mice (see Fig 3B, I), but not as drastic a loss as in Tg-HADH II/ABAD mice (see Fig 3D, I). Similarly, there were fewer MPTP-induced SNpc apoptotic neurons in Tg-HADH II/ABAD mice than in their wild-type counterparts (see Fig 3K, L).

Sparing of SNpc dopaminergic neurons does not always correlate with sparing of their corresponding striatal nerve fibers,[19] which is essential for maintaining dopaminergic neurotransmission. As seen in SNpc neuronal counts, the striatal optical density of TH immunostaining (see Fig 3E–H) in MPTP-injected mice was higher in Tg-HADH II/ABAD mice than in their wild-type littermates (see Fig 3J). In contrast, there were no differences in the extent of decline of striatal levels of dopamine and its main metabolites, DOPAC and HVA, between genotypes, after MPTP administration (Table 1). These findings indicate that HADH II/ABAD overexpression protects the nigrostriatal pathway structurally but not functionally against the toxicity of MPTP.

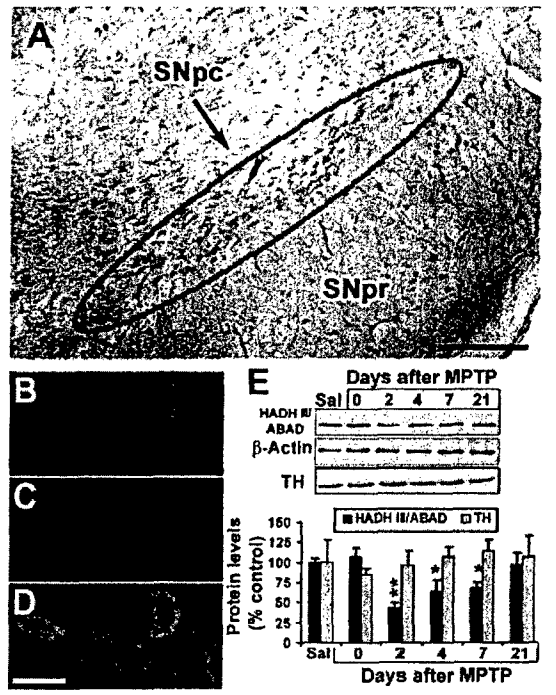

Fig 2. Expression of HADH II/ABAD in substantia nigra pars compacta (SNpc) tyrosine hydroxylase (TH)-positive neurons and its reduction in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–treated mice. Immunoreactivity of HADH II/ABAD is highly abundant in mouse ventral midbrain and the region of interest, substantia nigra (A). Analyses of double immunofluorescence confirm that HADH II/ABAD (B, green) colocalizes with TH-positive neurons (C, red) as demonstrated when they are merged (D, yellow). Scale bars = 250μm (A) and 25μm (B–D). In ventral midbrain samples, MPTP reduces levels of HADH II/ABAD but not TH proteins (E) at indicated time points. Protein levels were quantified as the ratio of HADH II/ABAD or TH over β-actin and expressed as percentage of their respective controls. Data represent mean ± SEM of 6 to 10 mice per group. (single asterisk) $p < 0.05$ *and* (double asterisks) $p < 0.01$ *compared with the control saline group.*

HADH II/ABAD Does Not Affect MPTP Activation

Striatal levels of MPP^+ 90 minutes after MPTP injection did not differ between Tg-HADH II/ABAD mice (29.26 ± 5.77μg/g striatal tissue, n = 4) and their wild-type littermates (30.40 ± 2.57, n = 6). Lack of difference between genotypes also was observed in the

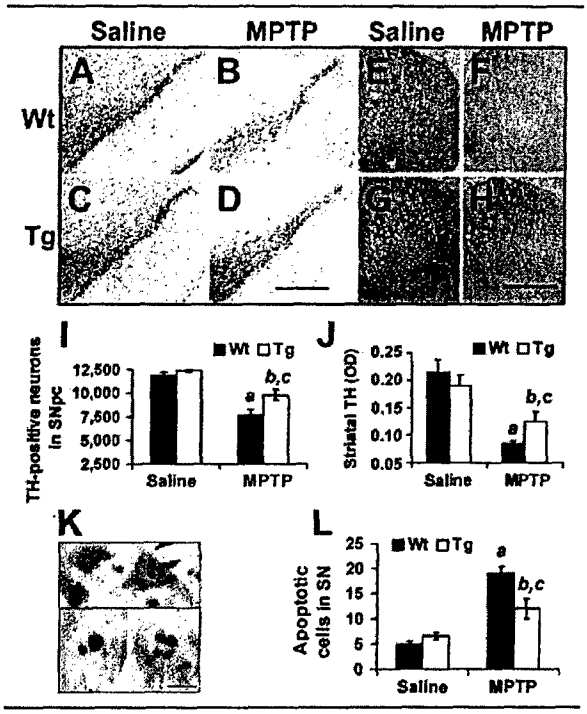

Fig 3. Protective effect of HADH II/ABAD against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced neurotoxicity. Overexpression of HADH II/ABAD significantly attenuates the loss of tyrosine hydroxylase (TH)-positive neurons (D, I) and terminals (H, J) as compared with their wild-type (Wt) counterparts (B, I) and (F, J). HADH II/ABAD also attenuates apoptotic cell death in substantia nigra (L) as evidenced by Nissl staining (K). Compared with normal neurons (K, top panel), these apoptotic cells appear as distinct, round, and well-defined cells as a result of chromatin condensation and shrinkage of cell body (K, bottom panels). Data represent mean ± SEM of three to six mice per group. $^{a}p < 0.001$ *compared with the Wt saline group,* $^{b}p < 0.05$ *compared with the transgenic (Tg) saline group.* $^{c}p < 0.05$ *compared with the Wt MPTP group. Scale bars = 500μm (A–D), 1mm (E–H), and 25μm (K).*

uptake of [^{3}H]-MPP$^+$ into striatal synaptosomes (IC_{50}, nM, wild type: 198.00 ± 35.05; Tg: 184.30 ± 20.11; n = 3) and in the accumulation of [^{3}H]-MPP$^+$ in mitochondria (wild type: 100.00 ± 8.29%; Tg: 103.51 ± 3.54 of wild-type control; n = 3). Thus, overexpression of HADH II/ABAD does not confer protection by altering MPTP metabolism or the entry of MPP$^+$ into dopaminergic neurons and subsequently into mitochondria, which are critical steps to the toxicokinetics of MPTP.[27]

HADH II/ABAD Increases Mitochondrial Respiration via Complex I

In mitochondria from wild-type mice, MPP$^+$ inhibited oxygen consumption mediated by complex I in a dose-dependent manner (Fig 4A). However, this inhibition was not as marked in mitochondria from Tg-HADH II/ABAD (see Fig 4A). At a high concentration of MPP$^+$ (100μM), in which approximately 90% of respiration was inhibited, there was no longer any difference in the magnitude of oxygen consumption inhibition between genotypes (see Fig 4A). A similar pattern also was observed when rotenone, a potent lipophilic complex I inhibitor, was used (see Fig 4B), suggesting that the effect of HADH II/ABAD on mitochondrial respiration is not solely restricted to MPP$^+$ but to inhibition of complex I in general. In addition, upon exposure to MPP$^+$, lactate production, an indirect measurement of MPP$^+$-induced complex I inhibition,[28] was lower in striatal tissues of Tg-HADH II/ABAD mice as compared with their wild-type counterparts (see Fig 4F). In well-coupled mitochondria, a higher rate of oxygen consumption correlates with a higher rate of ATP production.[29,30] Consistent with this, a higher rate of oxygen consumption in Tg-HADH II/ABAD mitochondria (see Fig 4A) was indeed associated with higher ATP levels (see Fig 4E).

In contrast to complex I inhibition, no differences between genotypes were detected in the rate of oxygen consumption mediated through complex II (see Fig 4C) and complex IV (see Fig 4D). Thus, these data suggest that upon partial inhibition of complex I, but not of other main electron transport chain enzymatic complexes, HADH II/ABAD can improve mitochondrial respiration and ATP production.

Overexpressing HADH II/ABAD Does Not Alter Mitochondrial Mass or Electron Transport Chain Complex Activities

To explore the mechanism by which HADH II/ABAD improves mitochondrial respiration, we first compared the mitochondrial mass between the two genotypes by measuring the enzymatic activities of citrate synthase, complex I, II, and IV in brain tissue homogenates (Table 2). None of these assays showed any differences between Tg and wild-type mice. Next, we asked whether

Table 1. Levels of Dopamine and Its Metabolites in Striatal Tissues (ng/mg)

	DA	DOPAC	HVA
Wt-saline	13.29 ± 1.41	3.66 ± 0.34	1.15 ± 0.09
Tg-saline	14.12 ± 0.94	3.06 ± 0.20	1.33 ± 0.09
Wt-MPTP	2.77 ± 0.26	1.30 ± 0.15	0.85 ± 0.03
Tg-MPTP	2.83 ± 0.40	2.29 ± 0.12	1.03 ± 0.02

Striatal levels of DA and its metabolites, DOPAC and HVA, were measured using high-performance liquid chromatography. No differences were detected between genotypes. Data represent mean ± SEM of four to five mice per group.

DA = dopamine; DOPAC = dihydroxyphenylacetic acid; HVA = homovanillic acid; Wt = wild type; Tg = transgenic; MPTP = l-methyl-y-phenyl-1,2,3,6-tetrahydropyridine.

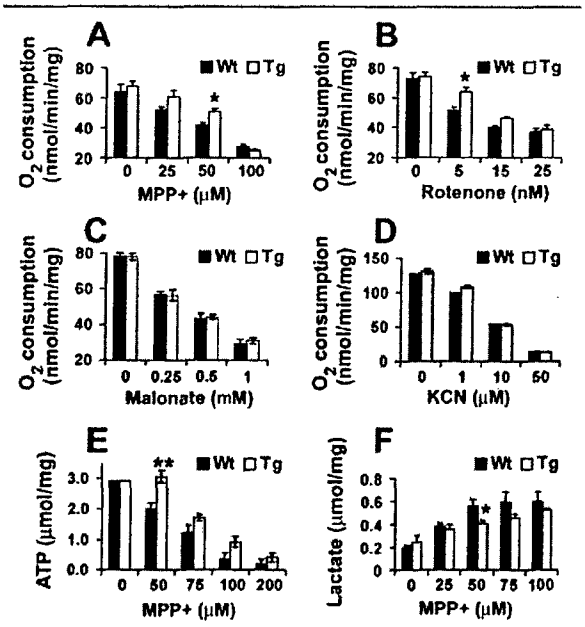

Fig 4. Improvement of mitochondrial functions in HADH II/ABAD mice. Oxygen consumption mediated through complexes I (A, B), II (C), and IV (D) was assessed in the presence or absence of their respective substrates or inhibitors as described in Material and Methods, using purified brain mitochondria. HADH II/ABAD attenuates mitochondrial inhibition specifically via complex I (A, B). Under these identical conditions, HADH II/ABAD also improves ATP production (E). Lactate production in striatal sections confirms MPP^+ has less of an inhibitory effect on mitochondria in transgenic (Tg)–HADH II/ABAD than in their wild-type (Wt) littermates. Data represent mean ± SEM of three to six mice per group, (asterisk) $p < 0.05$ *compared with their respective Wt control groups.*

overexpressing HADH II/ABAD induced permanent structural conformational changes in complex I, thereby reducing the potency of compounds such as MPP^+ or rotenone in blocking its activity. Using purified mitochondria to assess enzymatic activities, we did not detect differences in normal basal activities between Tg and wild-type mice for complexes I, II, or IV or citrate synthase (see Table 2). Because differences in oxygen consumption and ATP production in intact brain mitochondria between genotypes were seen only at certain concentrations of complex I inhibitors, the comparison of complex I enzymatic activity between Tg and wild-type mice was also performed in the presence of different concentrations of MPP^+ (Fig 5A) or rotenone (see Fig 5B). Under this system of opened mitochondria, there were no detectable differences in complex I inhibition at any of the MPP^+ or rotenone concentrations studied between wild-type and Tg-HADH II/ABAD mitochondria (see Fig 5A, B), even when large amounts of HADH II/ABAD recombinant protein were added to the medium (see Fig 5C). Together, these data indicate that, first, the effect of HADH II/ABAD on mitochondrial respiration is not caused by mitochondrial proliferation or complex I catalytic alteration and, second, intact mitochondria are required for HADH II/ABAD to mitigate the impact of complex I inhibition on mitochondrial respiration and ATP production.

Redistribution of HADH II/ABAD from Mitochondrial Matrix to Mitochondrial Membrane

To understand how HADH II/ABAD may sustain mitochondrial respiration under the metabolic stress caused by MPP^+ and rotenone, we assessed the intramitochondrial distribution of this enzyme. In normal respiring brain mitochondria, little HADH II/ABAD is found in the mitochondrial membrane fraction (Fig 6), which is to be expected because this is essentially a mitochondrial matrix enzyme. Conversely, upon incubation of brain mitochondria with MPP^+ or rotenone, there was an increase of HADH II/ABAD content in the mitochondrial membrane fraction. This translocation occurred specifically after complex I inhibition because it was not observed after complex II inhibition produced by malonate (see Fig 6). In contrast, the translocation of another mitochondrial matrix protein, heat shock protein 60 (HSP-60), was not detected (see Fig 6). The present data suggest that, upon complex I inhibition, HADH II/ABAD translocates from the mitochondrial matrix to the inner membrane, thereby allowing this enzyme to be in physical contact or in close

Table 2. Mitochondrial Mass and Activities of Individual Respiratory Chain Complexes (nmol/min/mg protein)

	Brain Homogenates		Brain Mitochondria	
	Wild Type	Tg-HADH II/ABAD	Wild Type	Tg-HADH II/ABAD
Complex I	13.73 ± 1.2	13.27 ± 1	46.5 ± 4.3	42.3 ± 3.6
Complex II	23.37 ± 2.9	21.6 ± 1.5	82.9 ± 1.7	84.0 ± 1.0
Complex IV	888 ± 51.1	951 ± 51.8	3,857.3 ± 67.3	4,145.1 ± 93.0
Citrate synthase	102 ± 13.6	105 ± 3.2	750.49 ± 16.5	796.06 ± 30.0

Brain homogenates and mitochondria were assessed as to whether overexpressing HADH II/ABAD alters the activities of respiratory chain complexes and mitochondrial mass in these animals. No differences were detected between genotypes. Data represent mean ± SEM of three to six mice per group.

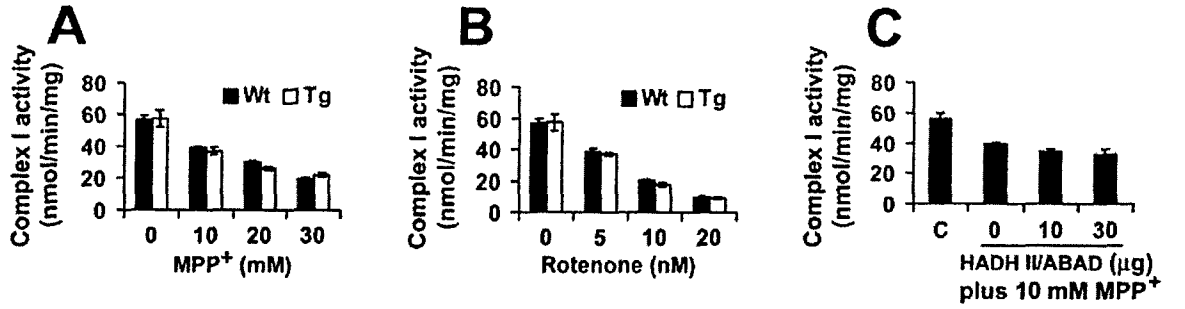

Fig 5. Lack of HADH II/ABAD effects in opened mitochondria. In lysed mitochondria, varying concentrations of MPP^+ (A) and rotenone (B) were used to induce different magnitudes of complex I inhibition like those seen in intact mitochondria in Figure 4. In this system, HADH II/ABAD failed to confer protection, despite the extra supplement of HADH II/ABAD recombinant protein (C). Data represent mean ± SEM of three mice per group.

proximity with complex I (which resides in the inner mitochondrial membrane).

HADH II/ABAD Is Not an Antioxidant

When MPP^+ binds to complex I, in addition to inhibition of mitochondrial respiration and ATP production, it also increases reactive oxygen species (ROS) production. The antioxidant effect of HADH II/ABAD was assessed in purified brain mitochondria obtained from Tg-HADH II/ABAD mice and their wild-type littermates. Table 3 shows that in the presence of MPP^+, HADH II/ABAD did not attenuate ROS production. HADH II/ABAD also did not attenuate ROS production induced by rotenone (data not shown).

Discussion

This study shows that HADH II/ABAD is constitutively expressed in all ventral midbrain neurons, including SNpc dopaminergic neurons, from normal human and mouse tissues. However, HADH II/ABAD tissue content is reduced in affected brain regions from PD patients and MPTP mice. We believe that the reduction of HADH II/ABAD in ventral midbrain in PD and MPTP-treated mice results, at least in part, from a downregulation of HADH II/ABAD and not solely from a loss of HADH II/ABAD-containing neurons for the following reasons. First, in MPTP-intoxicated mice, at the time points when HADH II/ABAD levels in ventral midbrains are reduced, TH levels, used as a phenotypic marker for dopaminergic neurons, are unaffected. The lack of detectable reduction in TH content by Western blot in the face of an approximately 40% reduction in TH-positive neuron numbers is likely caused by SNpc neurons contributing only to a small fraction of the total TH content in the ventral midbrain. Second, as mentioned above, HADH II/ABAD is expressed in all neurons, whereas MPTP only kills dopaminergic neurons. Third, the detection of HADH II/ABAD tissue content is reduced during the active phase of apoptosis, but not when cell loss is stabilized (21 days after MPTP treatment). Although we cannot rule out the possibility of compensatory upregulation of this enzyme at day 21 after MPTP injec-

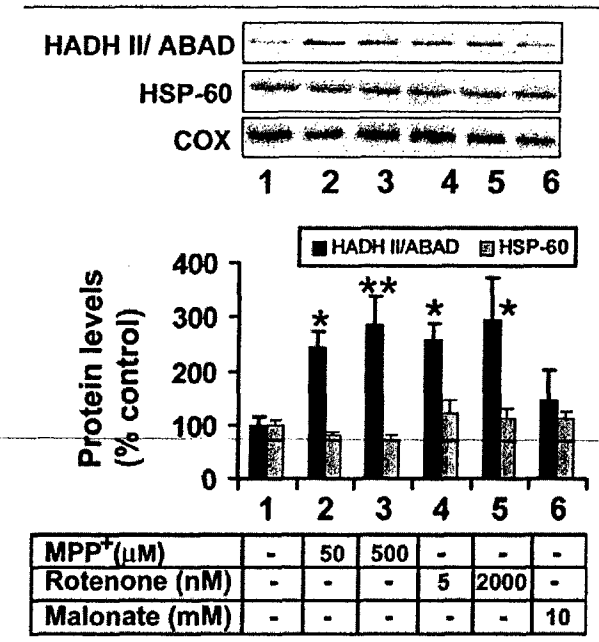

Fig 6. Translocation of HADH II/ABAD. Western blot analyses indicate that under the metabolic stress induced by the complex I inhibitors, MPP^+ or rotenone, HADH II/ABAD but not HSP-60 (another matrix protein) translocated from the matrix to the mitochondrial membrane. Malonate, a complex II inhibitor, failed to induce such an effect. Protein levels were quantified as the ratio of either HADH II/ABAD or HSP-60 over cytochrome c oxidase (COX) and expressed as percentage of their respective controls. Data represent mean ± SEM of six mice per group. (single asterisk) $p < 0.05$; (double asterisks) $p < 0.01$ *as compared with the control group (without inhibitor).*

Table 3. H_2O_2 Production in Purified Brain Mitochondria

Treatment	Mitochondrial H_2O_2 Production (pmol/min/mg protein)	
	Wild Type	Tg-HADH II/ABAD
Control	96.22 ± 12.47	119.09 ± 25.97
MPP^+ (50μM)	94.35 ± 5.0	71.62 ± 6.32
MPP^+ (500μM)	197.53 ± 23.97	160.18 ± 10.60

The fluorescence dye Amplex Red was used to measure H_2O_2 converted from superoxide. HADH II/ABAD does not have an antioxidant effect. Data represent mean ± SEM of three animals per group.

tions, we believe that the lower HADH II/ABAD immunoreactivity in the remaining SNpc dopaminergic neurons in PD tissues argues against this view. The downregulation of HADH II/ABAD in PD and MPTP tissues is not likely a result of a nonspecific alteration in dying neurons because other proteins such as BAX[21] and α-synuclein[31] are increased under these conditions.

Inactivation of the Drosophilae homolog of HADH II/ABAD, termed scully, leads to a lethal phenotype and developmental abnormalities,[32] and in *Caenorhabditis elegans*, reduction in the activities of the short-chain dehydrogenase-reductase family (of which HADH II/ABAD is a member), shortens life span.[33] These findings support a key role for HADH II/ABAD in cell survival and suggest that its downregulation may promote the demise of SNpc dopaminergic cells in PD and in the MPTP mouse model. Consistent with this view is our observation that more SNpc dopaminergic neurons survived in Tg mice overexpressing HADH II/ABAD compared with their wild-type littermates after MPTP administration. When we assessed apoptotic cell death under this MPTP regimen,[21] Tg mice exhibited less apoptotic cells than their wild-type counterparts. We demonstrated that the overexpression HADH II/ABAD was not associated with alterations in one of the key MPTP metabolic steps that determine its potency.[27]

ATP depletion is suspected to be important in MPTP neurotoxicity,[34] and interventions aimed at reducing the cellular energy demand or at increasing the cellular energy stores have all proved to effectively attenuate MPTP-induced neurodegeneration.[9,10,35] These studies highlight the importance of the ATP deficit in the MPTP neurodegenerative process. In normal rodents, dopaminergic structures represent a small fraction of all striatal cellular elements[36] and not much more in the ventral midbrain. This fact renders any reliable detection of ATP changes in brain tissues of MPTP-intoxicated mice[34] precarious. To avoid this problem, we studied the effects of HADH II/ABAD on ATP production and mitochondrial function in purified mitochondria. Through this approach, we demonstrated that mitochondria from Tg-HADH II/ABAD mice had higher oxygen consumption and ATP levels after exposure to MPP^+ compared with mitochondria from their wild-type littermates. These data indicate that overexpression of HADH II/ABAD allows the mitochondria to maintain a higher rate of oxidative phosphorylation and production of ATP in the face of a blockade of complex I. This benefit does not result from permanent structural alterations to the electron transport chain enzymes as evidenced by the lack of difference in complex I enzymatic activity between HADH II/ABAD genotypes. Our data also suggest that, for HADH II/ABAD to confer this protection, intact mitochondria are a prerequisite, perhaps to maintain the intricate interactions between the TCA cycle and the electron transport chain.

Our data further suggest that in active mitochondria, the metabolic stress induced by complex I inhibitors serves as a signal to recruit HADH II/ABAD to the mitochondrial membrane. In contrast, we failed to observe a similar pattern of migration with the mitochondrial matrix marker HSP-60. This latter finding indicates that the increased HADH II/ABAD level in the membrane fraction is not merely a result of nonspecific binding. Once relocated, HADH II/ABAD may increase complex I metabolic efficiency. The failure of HADH II/ABAD recombinant to mitigate the effects of MPP^+ and rotenone on complex I activity in opened mitochondria suggests that the functional interaction of HADH II/ABAD with complex I depends on more than just quantity and proximity.

The exact mechanism by which HADH II/ABAD confers neuroprotection through its suspected interaction with complex I requires further studies. Also, warranting additional investigations is the possible functional link between HADH II/ABAD and Poly(ADP-ribose) polymerase (PARP). This enzyme, which is a DNA binding protein that uses NADH as a substrate, happens to be activated after MPTP administration to mice.[35,37] Thus, HADH II/ABAD overexpression, by generating more NADH, could mitigate the depleting effect of PARP activation on the cellular stores of NADH. Because PARP activation is deleterious in the MPTP model of PD, this scenario may explain, at least in part, how overexpression of HADH II/ABAD confers neuroprotection in the MPTP model of PD.

Blockade of complex I by MPP^+ also stimulates the production of ROS, which have been implicated in inflicting serious oxidative damage to dopaminergic neurons.[27] Here, we have found no evidence that HADH II/ABAD overexpression abates ROS production. The partial nature of the protective effect mediated by HADH II/ABAD is likely due to the fact that while increased expression of this enzyme lessens MPP^+-mediated energy crisis, it does not curtail MPP^+-

mediated oxidative stress. Furthermore, as the rate-limiting enzyme in dopamine synthesis, TH is inactivated by oxidative stress after MPTP injection.[38] This inactivation of TH enzymatic activity may explain the lack of comparable sparing of striatal dopamine and its metabolites against MPTP. Accordingly, in diseases such as PD, where both energy crisis and oxidative stress are presumably instrumental in the neurodegenerative process, optimal neuroprotective strategies for PD may rely on the combination of energy stores-boosting agents and antioxidants.

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MPTP as a Mitochondrial Neurotoxic Model of Parkinson's Disease

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1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin extensively used to model Parkinson's disease (PD). A cascade of deleterous events, in which mitochondria play a pivotal role, drives MPTP neurotoxicity. How mitochondria are affected by MPTP and how their defect contributes to the demise of dopaminergic neurons in this model of PD are discussed in this review.

KEY WORDS: MPTP; Parkinson's disease; neurodegeneration; mitochondria; oxidative stress; ATP depletion; programmed cell death.

1-Methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects (Ziering *et al.*, 1947). MPTP can induce a parkinsonian syndrome in humans and nonhuman primates almost indistinguishable from Parkinson's disease (PD) on both clinical and neuropathological standpoints (Langston and Irwin, 1986). Over the years, MPTP has been used in a host of different animal species, especially in mice (Heiklla *et al.*, 1989), to recapitulate the hallmark of PD cellular pathology, namely the degeneration of the nigrostriatal dopaminergic pathway (Dauer and Przedborski, 2003). Although the MPTP model departs from PD on several significant aspects, it continues to be regarded as the best experimental model of this common neurodegenerative disease. With respect to PD, enthusiasm for the MPTP model is driven by the belief that unraveling the MPTP neurotoxic process in animals may provide hints into the mechanisms responsible for the demise of dopaminergic neurons in human PD.

Various key cellular and molecular components underlying the MPTP neurotoxic process have been reviewed in details in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) and will thus not be discussed here. Instead, the focus of this minireview will be devoted to the role of the mitochondria in the deleterious effects of the parkinsonian toxin MPTP.

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FIRST STEP FIRST

MPTP is a protoxin whose toxicokinetics is a complex, multistep process (Dauer and Przedborski, 2003). As indicated by its octanol/water partition coefficient of 15.6 (Riachi *et al.*, 1989), MPTP is a highly lipophilic molecule, which is able to readily permeate lipid bilayer membranes. It is therefore not surprising to observe that MPTP crosses the blood-brain barrier in a matter of seconds after its systemic administration (Markey *et al.*, 1984). Once in the brain, it is rapidly converted into 1-methyl-4-phenylpyridinium (MPP^+), the actual neurotoxin (Heikkila *et al.*, 1984). This critical transformation of MPTP into MPP^+ is a two-step process. First, MPTP undergoes a two-electron oxidation, catalyzed by monoamine oxidase B (MAO-B), yielding the intermediate 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) (Chiba *et al.*, 1984). Given the discrete cellular distribution of MAO-B in the brain (Kitahama *et al.*, 1991), it is believed that the conversion of MPTP to $MPDP^+$ occurs specifically in glial and serotonergic cells, and not in dopaminergic neurons. $MPDP^+$ is an unstable molecule which readily undergoes spontaneous

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disproportionation to MPP^+ and MPTP (Chiba *et al.*, 1985; Peterson *et al.*, 1985).

Once formed, MPP^+ is presumably released from glial and serotonergic cells into the extracellular space prior to entering dopaminergic neurons. Yet, MPP^+ has an octanol/water partition coefficient of 0.09 (Riachi *et al.*, 1989), which indicates that, while being a lipophilic cation, MPP^+ is far less lipophilic than MPTP. Thus, unlike MPTP, MPP^+ is most likely unable to easily diffuse across cellular lipid bilayer membranes. Instead, it is to be expected that the release of MPP^+ from its intracellular sites of formation and entry into adjacent neurons depend on specialized carriers. Consistent with this view is the fact that MPP^+ access to dopaminergic neurons relies on the plasma membrane dopamine transporter (Bezard *et al.*, 1999; Javitch *et al.*, 1985).

MITOCHONDRIAL ACCUMULATION

Once inside neurons, MPP^+ rapidly accumulates in the mitochondrial matrix (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). Initially, it was thought that MPP^+ gains access to the mitochondrial matrix through a carrier (Ramsay *et al.*, 1986; Ramsay and Singer, 1986). However, it is now well established that MPP^+ is passively transported (Davey *et al.*, 1992; Hoppel *et al.*, 1987) by a mechanism relying entirely upon the large mitochondrial transmembrane potential gradient ($\Delta\psi$) of -150 to -170 mV (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987; Ramsay *et al.*, 1986; Ramsay and Singer, 1986).

Like with other lipophilic cations (Rottenberg, 1984), the higher the concentrations of intramitochondrial MPP^+, the lower the $\Delta\psi$ and, consequently, the slower the uptake of extramitochondrial MPP^+ (Davey *et al.*, 1992; Hoppel *et al.*, 1987). The demonstration that the ion-pairing agent tetraphenylboron anion increases both the rate and the extent of MPP^+ uptake in isolated mitochondria (Aiuchi *et al.*, 1988; Davey *et al.*, 1992; Hoppel *et al.*, 1987) further supports this concept. As discussed below, MPP^+ inhibits mitochondrial respiration, which likely also contributes to the loss of the $\Delta\psi$ gradient and to the dampening of the mitochondrial uptake of MPP^+. It is thus not surprising that the accumulation of MPP^+ by energized mitochondria behaves as a saturable phenomenon in the presence of high extramitochondrial concentrations of MPP^+ (e.g., >10 mM) (Ramsay and Singer, 1986) and appears to reach a steady state after a few minutes (Davey *et al.*, 1992; Ramsay *et al.*, 1986). This apparent steady state persists until mitochondrial suspension becomes anaerobic or $\Delta\psi$ is collapsed by the addition of an uncoupler agent such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Ramsay *et al.*, 1986). Remarkably, energized mitochondria incubated with 0.5 mM MPP^+ reach matrix concentrations of more than 24 mM after only 10 min (Ramsay and Singer, 1986). This fast and avid uptake suggests that most, if not all, of the cytosolic MPP^+ would eventually accumulate in the mitochondrial matrix after the systemic injection of MPTP.

INTRAMITOCHONDRIAL MPP^+

It is well established that intramitochondrial MPP^+ inhibits oxidative phosphorylation (Nicklas *et al.*, 1985; Singer *et al.*, 1987). Intramitochondrial MPP^+ also appears to inhibit the tricarboxylic acid cycle enzyme α-ketoglutarate dehydrogenase (Mizuno *et al.*, 1987a). Although both mitochondrial metabolic alterations may contribute to MPP^+ cytotoxicity, attention has been paid almost exclusively to the action of MPP^+ on the respiratory chain.

It is well documented that MPP^+ impairs, in a dose- and time-dependent manner, the ADP-stimulated oxygen consumption (State 3) in intact mitochondria supported by the NADH-linked substrates glutamate and malate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). MPP^+ is, however, ineffective in inhibiting the oxygen consumption in mitochondria supported by succinate (Mizuno *et al.*, 1987b; Nicklas *et al.*, 1985). Furthermore, MPP^+ prevents the binding of the classical Complex I inhibitor [^{14}C]-rotenone to electron transport particles (Ramsay *et al.*, 1991a). Collectively these findings indicate that MPP^+, like rotenone and piericidin A, impairs mitochondrial respiration by inhibiting the multi-subunit enzyme Complex I (i.e., NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain. This straightforward interpretation is supported by the electron spin resonance demonstration that MPP^+ does actually bind to Complex I and blocks the terminal step of electron transfer from the highest potential iron–sulfur cluster of Complex I called N2 to ubiquinone (Ramsay *et al.*, 1987).

The use of several MPP^+ analogs and cationic inhibitors has demonstrated that MPP^+ binds at two distinct sites within the mitochondrial electron transport chain region comprised between N2 and ubiquinone (Gluck *et al.*, 1994; Miyoshi *et al.*, 1997, 1998; Ramsay *et al.*, 1989, 1991b; Ramsay and Singer, 1992). These studies have also demonstrated that the occupation of both sites appears to be required for complete inhibition of NADH oxidation. The binding of MPP^+ to the first, more *hydrophilic* site seems to primarily affect the functional coupling between the PSST and the ND1 subunit of Complex I and to account

for only 40% of the MPP^+-induced reduction in NADH oxidation (Schuler and Casida, 2001). The binding of MPP^+ to the second, more *hydrophobic* site seems quite potent in blocking Complex I enzymatic activity (Schuler and Casida, 2001). Yet, the exact location of this second binding site in Complex I remains to be determined. Nonetheless, the importance of the binding to PSST, but not to the ND1 subunit in the inhibition of Complex I-mediated NADH oxidation (Schuler *et al.*, 1999; Schule and Casida, 2001), suggests that the MPP^+ *hydrophobic* site must also be situated somewhere in the PSST subunit. This *hydrophobic* site appears not to exist for other typical Complex I inhibitors such as rotenone and piericidin A (Schuler and Casida, 2001). Accordingly, while MPP^+ binds to Complex I, as do rotenone and piericidin A (Gluck *et al.*, 1994; Krueger *et al.*, 1993), it may not bind to exactly the same Complex I subunit or subunit part as these two other Complex I inhibitors. Also worth noting is the fact that MPP^+, compared to rotenone and piericidin A, is a far weaker inhibitor of Complex I, which may explain why millimolar concentrations of MPP^+ are needed to inhibit NADH-oxidation in electron transport particles (Hoppel *et al.*, 1987).

CONSEQUENCES OF MPP^+-INDUCED COMPLEX I INHIBITION

In response to MPP^+ binding to Complex I, the flow of electrons along the respiratory chain is hampered in both dose- and time-dependent manners (Hasegawa *et al.*, 1990; Nicklas *et al.*, 1985; Vyas *et al.*, 1986). The importance of the inhibition of Complex I in the MPTP-induced neurotoxicity in vivo is supported by the demonstration that strategies aimed at stimulating oxidative phosphorylation via by-passing the blockade of Complex I not only improve mitochondrial respiration but also mitigate dopaminergic neurodegeneration in mice (Tieu *et al.*, 2003).

The current hypothesis on MPTP cytotoxicity posits that one of the main contributors to cell death is the impaired synthesis of ATP resulting from the inhibition of Complex I by MPP^+. Relevant to this view is the fact that MPP^+ indeed causes a rapid and profound depletion of cellular ATP levels in isolated hepatocytes (Di Monte *et al.*, 1986), in brain synaptosomal preparations (Scotcher *et al.*, 1990), and in whole mouse brain tissues (Chan *et al.*, 1991). It appears, however, that Complex I activity should be reduced by more that 50% to cause significant ATP depletion in nonsynaptic brain mitochondria (Davey and Clark, 1996). Furthermore, in vivo MPTP causes only a transient 20% reduction in mouse striatal and midbrain ATP levels (Chan *et al.*, 1991). These facts argue against MPP^+-related ATP deficits being the sole factor underlying MPTP-induced cell death.

Another consequence of Complex I inhibition by MPP^+ is an increased production of reactive oxygen species (ROS). It was shown that incubation of MPTP with brain mitochondria resulted in an oxygen-dependent formation of ROS (Rossetti *et al.*, 1988). It was also shown that incubation of MPP^+ with bovine heart submitochondrial particles causes a production of superoxide radicals when MPP^+ is used at the concentrations expected to be found inside neurons after MPTP systemic administration (Hasegawa *et al.*, 1990). In this study, the authors also demonstrate that the degree of Complex I inhibition is proportional to the amount of superoxide radical produced (Hasegawa *et al.*, 1990). Because modulations of key mitochondrial ROS scavengers, such as manganese superoxide dismutase, affect MPTP-induced neurotoxicity in mice (Andreassen *et al.*, 2001; Klivenyi *et al.*, 1998), it is reasonable to assert that MPP^+-related ROS production also contributes to MPTP-induced cell death.

CONCLUSION

As discussed above, ATP depletion and ROS overproduction appear to occur soon after MPTP injection, subjecting the intoxicated cells, early on, to an energy crisis and oxidative stress. However, the time course of these perturbations reviewed in the following reference (Przedborski and Vila, 2003) appears to correlate poorly with the time course of neuronal death in vivo (Jackson-Lewis *et al.*, 1995). What this meta-analysis is suggesting is that only a few neurons are probably succumbing to the early combined effects of ATP depletion and ROS overproduction. Instead, mounting evidence discussed in the following references (Dauer and Przedborski, 2003; Przedborski and Vila, 2003) indicates that rather than killing the cells, alterations in ATP synthesis and ROS production are pivotal in triggering cell-death-related molecular pathways which, once activated, rapidly lead to the demise of the intoxicated neurons.

Interestingly enough, among these latter molecular pathways, it appears that the mitochondrial-dependent programmed cell death machinery plays a critical role (Vila *et al.*, 2001). As illustrated in Fig. 1, it is thus plausible that the death of neurons caused by MPTP results from a circular cascade of deleterious events starting at the mitochondria by the alteration of the oxidative phosphorylation and finishing also at the mitochondria by the activation of the programmed cell death machinery. Whether the whole circuit depicted above is entirely orchestrated

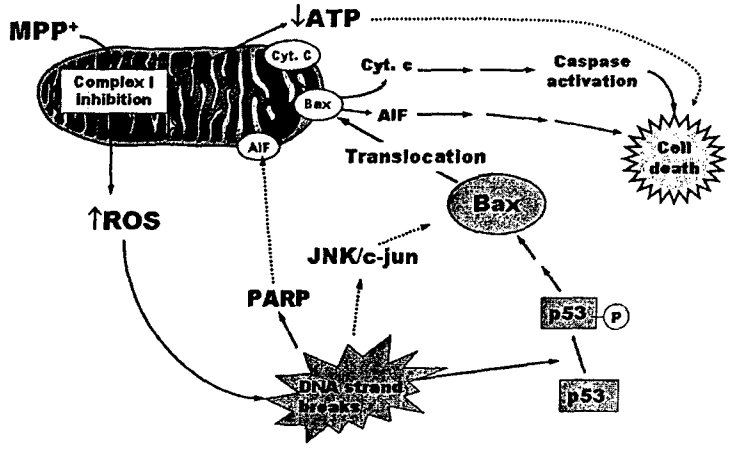

Fig. 1. Illustration *of the proposed circular nature of the MPP+-mediated cell death cascade* MPP^+ enters in the mitochondrion and binds to Complex I, whereby it inhibits ATP synthesis and stimulates ROS production. These two initial events lead to a host of cellular perturbations such as DNA damage, which, in turn, trigger a variety of cell-death-related pathways. These include activations of p53 by phosphorylation (p53-*p*) and JNK/c-Jun, which lead to Bax induction and translocation to the mitochondria. DNA damage also stimulates poly(ADP-ribose) polymerase (PARP) activity. Bax translocation and PARP activation promote the translocation of cytochrome *c* and apoptosis-inducing factor (AIF) from the mitochondria to the cytosol. Once in the cytosol, cytochrome c participates in a caspase-dependent cell death process, while AIF participates in a caspase-independent cell death process, both of which are not necessary mutually exclusive. Solid arrow, known mechanism; dashed arrow, speculated mechanism.

at the level of the mitochondria or whether it also involves perturbations that arise in the cytosol (e.g., protein nitration, cyclooxygenase-2 induction) and the nucleus (e.g., DNA damage, PARP activation) of the intoxicated cells is the focus of several ongoing studies in our laboratory.

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APPENDIX 7

CHAPTER

B3

From Man to Mouse: The MPTP Model of Parkinson Disease

VERNICE JACKSON-LEWIS and RICHARD JAY SMEYNE

In 1817 Dr. James Parkinson first described the syndrome that we know today as Parkinson disease (PD) in a paper entitled "An Essay on the Shaking Palsy" (Parkinson 1817). PD is a debilitating neurological disorder that strikes approximately 1–2% of the adult population older than fifty years of age (new incidence is 20 per 100,000 persons) (de Rijk et al. 1995). Current estimates from the American Parkinson Disease Foundation put the number of American citizens suffering from this disease at greater than one million persons. The costs of treatment of PD can be staggering. At an average per patient cost of $6,000 per year—for drugs, physicians, and loss of pay to patient and family members—(Whetten-Goldstein et al. 1997), the total cost of the disease may approach $6,000,000,000 per year; of which 85% is borne by private and government (e.g., Social Security, Medicare) insurance. In fact, more individuals present with PD than with multiple sclerosis, muscular dystrophy, and amyotropic lateral sclerosis (Lou Gehrig disease) combined (The Parkinsons Web, 1997). Since the population of the world is, on average, getting progressively older (United States Census Bureau, 1996), the number of people suffering from this disease should increase substantially within the next several decades. Furthermore, PD is an incurable disease with an average life expectancy after diagnosis of over fifteen years, thus there should be an even larger burden on both the social and financial resources of families, insurance companies, and the federal government than is present today.

I. BACKGROUND

Parkinson disease is characterized by a loss of the pigmented cells located in the midbrain substantia nigra pars compacta (SNpc). The loss of these cells results in a reduction in afferent fibers that project to the striatum. PD symptoms first manifest when approximately 60% of the SNpc neurons have already died (German et al. 1989). Because the progression of cell loss is thought to occur over a somewhat protracted period of time in a defined spatiotemporal manner (Damier et al. 1999; Nurmi et al. 2001), the onset of Parkinson disease symptoms is often insidious.

The underlying cause for the vast majority of PD cases is unknown. Controversy still exists as to how much of the disease results from a strict genetic causation, a purely environmental factor, or the more parsimonious combination of the two risk factors (Duvoisin 1999; Williams et al. 1999; Gasser 2001). Empirical evidence suggests that less than 10% of all diagnosed Parkinsonism has a strict familial etiology (Payami and Zareparsi 1998). A small number of

familial parkinsonian patients appear to have polymorphisms in the α-synuclein gene (Polymeropoulos et al. 1997), suggesting that this aggregating protein (Spillantini et al. 1997) may play a role in Lewy body formation that ultimately results in substantia nigra cell death (Nussbaum and Polymeropoulos, 1997). A second autosomal recessive locus coding for the parkin protein maps to the long arm of chromosome 6 (6q25.2-q27). Mutations in this gene cause a form of juvenile onset PD. Other genes that are associated with PD include loci at human chromosome 2p13 and 4p (Gasser, 2001). The PD linked to this locus more closely resembles that of idiopathic PD, although like the α-synuclein protein, this unknown protein has very low penetrance. However, at this time no mutations in these proteins are reported in idiopathic PD (Hu et al. 1999; Scott et al. 1999).

Because the vast majority of PD patients have no direct tie to any identified genetic mutation, important information regarding the pathophysiology of PD may be gleaned through the study of animal models. Several animal models have examined the mechanism(s) underlying the pathophysiology of experimental PD, including surgical and chemical models. One of the earliest models made use of a lesion of nigrostriatal pathway in which fibers emanating from the substantia nigra proceedings to the striatum rostrally through the medial forebrain bundle (Faull and Mehler 1978; Levine et al. 1983; Brecknell et al. 1995). In addition to the physical lesion studies, chemical lesions have also modeled Parkinson disease. In these studies, animals were injected with 6-OHDA, a neurotoxin that when injected into the striatum causes a retrograde degeneration of dopaminergic neurons in the SNpc (reviewed in Olney et al. 1990; Schwarting and Huston 1996; Deumens et al. 2002; Hirsch et al. 2003). A third model of experimental PD utilizes the properties of selective neurotoxins, the most famous of which is the loss of SNpc neurons following administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

II. MPTP

The discovery of MPTP has provided a useful model of Parkinsonism that appears to recapitulate the pathology of the disease seen in humans. The identification of MPTP may be one of the few cases in which a specific neurotoxin was discovered in humans first, followed by development of an animal model. The story first started around 1976 when a chemistry student named Barry Kidston was synthesizing a "designer" heroin, MPPP, for recreational use. Although generally successful, at one point he hurried the catalysis of the procedure and instead of producing MPPP, he synthesized a neurotoxin that a team from the National Institutes of Mental Health later found to be MPTP. After IV injection of the incorrectly designed drug, Kidston quickly exhibited a severe bradykinesia. Following a rapid hospitalization and initial diagnosis as a catatonic schizophrenic, physicians eventually suspected that he had an acute form of Parkinson disease. Kidston's symptomatic recovery after he was administered L-dopa confirmed suspicions. Because this was an isolated case, the details never attained public prominence, but this changed in the early 1980s after a number of northern California heroin users were identified who presented at various emergency rooms with symptoms indistinguishable from those of Parkinson disease (Burns et al. 1985; Langston 1985). The potential threat of a public health risk that could have been epidemic, brought the case of these "frozen addicts" to public awareness (Langston 1985). A complete history of these cases is presented in the book *The Case of the Frozen Addicts* (Langston and Palfreman 1996) as well as in the NOVA documentary of the same name (original broadcast date: February 18, 1986).

In the subsequent years since MPTP was identified in humans as a Parkinsonian agent, researchers have demonstrated that MPTP exerts its neurotoxic effects in a number of other primates (Kopin and Markey 1988; Jenner 2003; Wichmann and DeLong 2003), as well as in cats, and in several rodents. In rodents, only specific strains of mice are sensitive to the administration of MPTP (Sundstrom et al. 1987; Riachi and Harik 1988; Mitra et al. 1994; Hamre et al. 1999). MPTP structurally resembles several known environmental agents, including well-known herbicides such as paraquat (Di Monte et al. 1986) and garden insecticides and fish toxins such as rotenone (McNaught et al. 1996) that induce dopamine cell degeneration (Brooks et al. 1999; Betarbet et al. 2000; Thiruchelvam et al. 2000; Chun et al. 2001). As such, it is possible, although as of yet unproven, that the genetic pathways and mechanisms that underlie the toxin-induced cell death of each of these compounds may interact.

There are many points systemically where MPTP can affect the dopaminergic system (Figure 1). In this chapter, we will discuss each step in the MPTP toxification pathway.

Step 1. Introduction of MPTP into the CNS

MPTP, in and of itself, is not toxic. The enzyme MAO-B metabolizes MPTP to the unstable 1-methyl-4-phenyl-2, 3-dihydropyridium ($MPDP^+$) that then rehydrogenates or deprotonates to generate MPTP or the corresponding pyridium species, MPP^+, respectively (Figure 2). At the point of interface with the periphery, exogenous compounds can either enter or be excluded from the CNS by the blood-brain barrier (BBB). The BBB is composed on tight-junctioned endothelial cells that make up the microvasculature of the brain in tight opposition with the end feet of glial processes. Endothelial cells of the microvasculature contain monoamine oxidases, and several studies have correlated levels of monoamine oxidases with MPTP-induced neuronal loss (Kalaria et al. 1987; Riachi et al. 1988). Since MPP^+

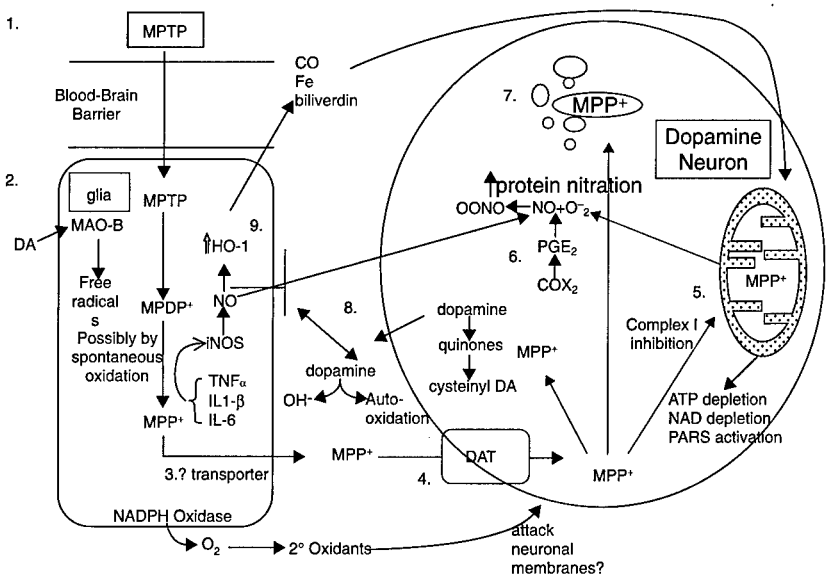

FIGURE 1 Proposed mechanism of MPTP action in the substantia nigra and striatum. The numbers represent each step in the toxification process outlined in this chapter.

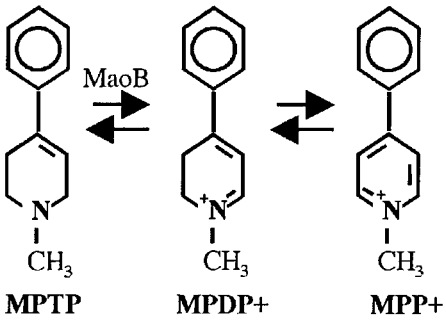

FIGURE 2 The protoxin MPTP is converted by monoamine oxidase B (MaoB) through intermediates to the toxin MPP^+.

cannot be transported through the BBB (Riachi et al. 1990), this level of toxification/detoxification can provide a first line of defense against exogenous agents.

Step 2. Role of Glia in the Toxification of MPTP

MPTP that is not deprotonated to MPP^+ rapidly enters the brain and is taken up into glial cells by a number of mechanisms including monoamine (Brooks et al. 1989) and glutamate (Hazell et al. 1997) transporters or pH-dependent antiporters (Kopin 1992; Marini et al. 1992). Glia, like endothelial cells, also contain large pools of monoamine oxidases and also convert MPTP from its protoxin form to MPP^+ (Ransom et al. 1987), in a manner dependent on the presence of MAO-B. A study by Brooks et al. (1989) provided additional support for the role of glial cells in dopaminergic neuronal toxicity, demonstrating that administration of fluoxetine (a serotonergic uptake inhibitor) immediately before systemic injection of MPTP attenuated neurotoxicity. Because fluoxetine did not alter the neurotoxicity of injected MPTP, the site of activation was proven to be extraneuronal, lending credence to the observation that the primary step in MPTP toxicity occurred in the astrocyte.

Once converted to MPP^+ in the astrocyte, MPP^+ stimulates the up-regulation of TNF-alpha, interleukin-1-beta (IL-1β) and interleukin-6 (IL-6) (Youdim et al. 2002; Teismann et al. 2003a) and these, in turn, up-regulate inducible nitric oxide synthese (iNOS) (Hunot et al. 1999). Of the three NOS isoforms present in the brain, endothelial NOS (eNOS), found mainly in the vasculature of the brain, does not contribute to MPTP toxicity (Wu and Przedborski, personal communication). In addition, since neuronal NOS (nNOS) knock-out mice show partial protection against the disastrous effects of MPTP administration (Przedborski et al. 1996) in the substantia nigra pars compacta (SNpc), another NOS isoform must also contribute to the neurotoxicity of MPTP. iNOS, a NOS isoform that is minimally expressed in the brain in non-pathological conditions, is highly expressed in the substantia nigra in both Parkinson disease (PD) and in mice (most likely in the microglia) following MPTP treatment (Hunot et al. 1996; Liberatore et al. 1999; Wu et al. 2002; Wu et al. 2003). iNOS produces large amounts of nitric oxide (NO), which is an uncharged, lipophilic molecule (Lancaster 1996) that can freely pass through membranes and travel distances greater than the length of a neuron, up to 300 microns, to do its damage remotely. Thus, under pathological conditions or following MPTP treatment, neurons in the vicinity of the NO molecule are at risk for possible attack by glial-derived reactive nitrogen–related

species. Because minocycline, a second-generation tetracycline antibiotic, can block iNOS induction (Wu et al. 2002), this step in the toxification process of MPTP presents a point where potential therapeutics may have a significant impact.

Step 3. Release of MPP^+ from Glia

MPP^+ is a polar compound and as such cannot freely exit glial cells. The question of how this compound exits the cell is currently under investigation. Investigators speculate that a specific transporter may actively move this polar molecule out of the glia (Russ et al. 1996; Inazu et al. 2003), however, at present, the specific mechanism remains unknown.

Step 4. Transport of MPP^+ into the Dopaminergic Neuron

Once released into the extracellular space, upon encountering neurons, MPP^+ is taken up into the cell preferentially by the dopamine transporter (DAT). *In situ* analysis has shown that the midbrain contains the highest concentration of dopamine transporters/cell (Cerruti et al. 1993). For this reason, as well as for the selectivity of dopaminergic neurons to many exogenous compounds, the DAT may be a control point in determining differential susceptibility to agents that are known to damage midbrain neurons (Kitayama et al. 1993; Le Couteur et al. 1997) (but see also the study by Higuchi et al. 1995). Two groups demonstrated the absolute necessity for the DAT in MPTP toxicity when they examined mice carrying null mutations of the DAT (Gainetdinov et al. 1997; Bezard et al. 1999). In these studies, MPTP-susceptible strains of mice carrying null mutations of the DAT were completely protected from MPTP toxicity.

Step 5. Effects of MPP^+ on Mitochondria within Dopaminergic Neurons

Once in the cell, MPP^+ has several paths: it can enter into mitochondria (step 5) where it interferes with complex I of the electron transport chain (Nicklas et al. 1987; Lander and Schork 1994) or it can be sequestered into cytoplasmic vesicles through the vesicular monoamine transporter (see step 7) (Liu et al. 1992; Del Zompo et al. 1993). Both of these steps have been implicated in processes that either protect or kill the dopaminergic neurons.

MPP^+ enters the mitochondria by the diffusion of this lipophilic cation through the mitochondrial inner membrane. The uptake of MPP^+ into mitochondria is not passive but is actively driven by an electrical gradient within the membrane (a Km of about 5 mM). This active transport was supported by experiments in which valinomycin plus K^+, which collapses the electrochemical mitochondrial gradient, abolished MPP^+ uptake, while agents that specifically collapsed the proton gradient had no effect on MPP^+ uptake (Ramsay et al. 1986; Ramsay and Singer 1986).

Once in the mitochondria, MPP^+ has been implicated in significant alterations of mitochondrial function. MPP^+ inhibits cellular respiration by blocking the mitochondrial electron transport enzyme NADH:ubiquinone oxidoreductase (complex I) (Nicklas et al. 1985; Suzuki et al. 1990) leading to a reduction in cellular ATP. Although this appears to be the major step in blocking mitochondrial function, studies also demonstrate that MPP^+ can directly inhibit complexes III (ubiquinol:ferrocytochrome c oxidoreductase) and IV (ferrocytochrome c:oxygen oxidoreductase or cytochrome c oxidase) of the electron transport chain (Mizuno et al. 1988a,b). The loss of cellular energy has several consequences, including the generation of the oxygen free radicals that rearrange to form hydrogen peroxide. Further catalysis leads to the formation of hydroxyl radicals.

The energy depletion due to MPP^+'s interference with complex I-III has led to a number of potential therapies. One of the most interesting is the use of Coenzyme Q10 supplementation, as several studies show that orally administering this enzyme can slow the progression of idiopathic PD (Beal 2003; Muller et al. 2003; Shults 2003).

Step 6. Role of Nitration within Dopaminergic Neurons

Although complex I inhibition by MPP^+ is known to reduce the energy production within dopaminergic neurons, it is possible, if not likely, that this is not the direct cause of the observed neuronal death. The damage done within SNpc neurons likely results from compounds generated in the cell, secondary to energy depletion. The formation of the superoxide radical is one example of this process. To establish the role of the superoxide radical in the MPTP neurotoxic cascade of events, a study by Cleeter et al. (1992), showed that MPP^+ inhibits mitochondrial complex 1 activity, which causes an excessive amount of superoxide radicals to form within the neuronal cytosol. Further support came from a study by Przedborski et al. (1992), which demonstrated that over-expression of the copper-zinc form of superoxide dismutase in mice is neuroprotective against the damaging effects of MPTP. Moreover, research by Wu et al. (2003), using the fluorescent tag hydroethidium, provided an *in vivo* demonstration of the presence of the superoxide radical in the MPTP neurotoxic process.

NO, produced in the glial cells, can enter the cytosol of the neuron via simple membrane diffusion. Neither the superoxide radical nor NO are particularly damaging by themselves; however, when the two interact, peroxynitrite ($OONO^-$), one of the most destructive oxidizing molecules, is formed (Ischiropoulos and al-Mehdi 1995; Przedborski

et al. 2000; Przedborski and Vila 2003). This fast-moving molecule as a single entity is hard to detect, however, its handiwork, the nitration of the tyrosine residues of a number of cellular components that include enzymes, transmitters, proteins, fatty acids, and DNA (Radi et al. 2002), can readily be documented.

While many molecules are affected by peroxynitrite, this chapter concentrates on intracellular proteins that are affected both in PD as well as in the MPTP mouse model. One potential target is tyrosine hydroxylase (TH), which is the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects its dense TH-positive fibers to the striatum (Grofova 1979). Because the cell-body rich SNpc contains primarily the soluble form of the TH enzyme, TH is often used as a faithful phenotypic marker for dopaminergic neuron numbers as well as an indicator of dopaminergic neuron loss (Jackson-Lewis et al. 1995).

TH is a tetrameric enzyme composed of four identical subunits. Each subunit carries catalytic activity, and catalytic domains have been localized to the carboxy terminals between leucine residue 188 and phenylalanine residue 456. While rodent (rat and mouse) TH contains seventeen tyrosine residues of which fifteen are in the catalytic domain, human TH, although similar, contains only fifteen tyrosine residues of which fourteen are in the catalytic domain (Saadat et al. 1988; Daubner et al. 1993). Tyrosine residues are the keys to the inactivation and nitration of TH, as they are the targets of nitration. At present, researchers speculate that Tyr225 is the most important residue because it lies within the sequence that is targeted for nitration (Przedborski and Jackson-Lewis 1998).

In PD, clinical symptoms appear when about 60–70% of the TH-positive cells in the SNpc have degenerated (Fahn and Przedborski 2000). In addition to the cell loss, brains of Parkinsonian patients show deficits in TH enzyme activity (Ara et al. 1998). Both *in vitro* and *in vivo* studies demonstrate that peroxynitrite impairs TH activity. In mice treated with MPTP, TH nitration seems to occur as early as three hours after MPTP administration. Immunoprecipitation studies using striata from MPTP-treated mice confirm that TH is indeed the nitrated protein. Furthermore, transgenic mice that overexpress human SOD do not show any detectable levels of nitrated striatal TH following MPTP treatment (Ara et al. 1998). Mice deficient in iNOS show less ventral midbrain nitrotyrosine, a fingerprint for tyrosine nitration, after MPTP administration than in their wild-type counterparts (Liberatore et al., 1999). Thus, the inactivation of TH via its nitration following exposure to both peroxynitrite and MPTP is important to the development of PD in humans and to the MPTP neurotoxic process in mice.

Dopamine (DA) is a relatively unstable molecule that is subjected to hydroxyl radical attack (Slivka and Cohen 1985) and autooxidizes in the extracellular space (Hirrlinger et al. 2002). In addition, dopamine can be nitrated within the neuron (LaVoie and Hastings 1999) and therefore may contribute to the degeneration of the cells that contain it as a transmitter. Here, DA is oxidized to DA quinone, which then undergoes a nucleophilic addition via the sulfhydryl group from cysteine, forming 5-cysteinyl-DA (Graham 1978). In pathological situations, the up-regulation of the cyclooxygenase-2 (COX-2) enzyme facilitates the oxidation of DA to 5-cysteinyl-DA (Hastings 1995; O'Banion 1999). The relationship of 5-cysteinyl-DA to neurodegeneration in PD as well as to the degeneration of DA neurons seen in the MPTP mouse model was recently investigated using a combination of immunocytochemistry (PD brains) or a combination of immunocytochemistry and HPLC (MPTP studies). In both PD brains and ventral midbrain from MPTP-treated mice, COX-2 enzyme activity and protein levels were significantly higher than in controls. Robust COX-2 immunostaining was also noted in both the human and mouse brains where the enzyme appeared to be confined to the cytosol of dopaminergic neurons (Teismann et al. 2003b). Furthermore, inhibition of the COX-2 response to MPTP prevented the rise in protein cysteinyl dopamine that occurred in mice following the administration of MPTP (Teismann et al. 2003b).

Peroxynitrite is formed from NO and the superoxide radical inside the neuron, and as such poses a serious threat to intracellular components such as mitochondria. For example, the nitration of manganese SOD (MnSOD), the primary mitochondrial antioxidant, was detected both *in vitro* (Quijano et al. 2001) and during inflammatory responses *in vivo* (MacMillan-Crow et al. 1996; Aulak et al. 2001). Here, nitration was proven to be site-specific in that it is tyrosine 34 (Tyr^{34}) among the tyrosine residues that is nitrated. In the MPTP mouse model, over-expression of human MnSOD localized to mitochondria prevented the accumulation of 3-nitrotyrosine, the faithful fingerprint of peroxynitrite-mediated nitration (Klivenyi et al. 1998). Several other mitochondrial components such as NADH: ubiquinone reductase (Complex I) (Riobo et al. 2001), cytochrome c (Cassina et al. 2000), aconitase, ATPase and VDAC (voltage dependent anion channel) (Radi et al. 2002) are nitrated following exposure to peroxynitrite. Whether these are nitrated in PD and in the MPTP mouse model has yet to be determined.

Step 7. Sequestration of MPP^+ within the Dopaminergic Neuron

The vesicular monoamine transporter VMAT2, is a proton-dependent transporter that sequesters monoamine neurotransmitters from free cytoplasmic space into synaptic vesicles (Miller et al. 1999a). Like the monoamines, MPP^+ can be transported by the VMAT into these vesicles, and as such, can be prevented from entering the mitochondria

where it can inhibit complex I. Investigators postulate that this sequestration may be a mechanism for attenuating the effects of any number of monoaminergic toxins. Support for this hypothesis comes from analyses in mice containing partial or complete deletions of VMAT2 and from human studies of VMAT expression. In parkinsonian humans, immunocytochemical localization of VMAT demonstrates reduced expression in striatum, paralleling the reductions seen in DAT. In fact, the relative expression of VMAT2, compared to that of DAT, may allow one to predict if and which dopamine neurons may be lost in PD (Miller et al. 1999b). In animal studies, mice heterozygous for VMAT2 and exposed to MPTP were examined for markers of dopaminergic neuron toxicity, including striatal dopamine content, the levels of DAT protein, as well as for a secondary marker of neurotoxicity, the expression of glial fibrillary acidic protein (GFAP) mRNA. In all parameters measured, VMAT2 +/- mice were more sensitive to MPTP-induced toxicity than their wild-type littermates (Gainetdinov et al. 1998). Further examination of these mice revealed that heterozygous VMAT2 mice, in addition to the loss of striatal markers, also had increased SNpc cell loss following administration of MPTP (Takahashi et al. 1997). These studies suggested an important role for VMAT2 in potentiating the effects of MPTP. Conversely, cells transfected to overexpress a greater density of VMAT2 were converted from MPP^+ sensitive to MPP^+ resistant cells (Liu et al. 1992). These studies suggested an important role for VMAT2 in potentiating or allaying the effects of MPTP.

Alpha-synuclein is another molecule relevant to the development of PD in humans and to the neurotoxic process in the MPTP mouse model of PD that is susceptible to nitration because of the presence of tyrosine residues. Historically, synucleins are vertebrate-specific cytosolic proteins that contain about 127–140 residues that have a unique 11-residue repeat that occurs in five to seven copies, accounting for roughly half of their structure and no structural domains. Four proteins, alpha, beta, and gamma synuclein and synoretin make up this family of proteins. Only two proteins in this family, alpha and beta, are synthesized in relatively large amounts in the brain and are highly expressed in presynaptic nerve terminals (Schluter et al. 2003). Synucleins account for about 1% of brain proteins and to date their functions are still unknown. Mutations in alpha-synuclein are associated with a familial form of PD (Polymeropoulos et al. 1997) that is readily indistinguishable from the more common sporadic form of the disease. The interaction between WT alpha-synuclein and mutant alpha-synuclein may enhance the ability of the different alpha-synucleins to interact with other cellular proteins to form aggregates (Conway et al. 1998).

One of the hallmarks of PD is the presence of Lewy bodies within neurons in the SNpc. Lewy bodies are both ubiquitin and alpha-synuclein positive. Since alpha-synuclein is the only synuclein present in Lewy bodies, it has to be determined whether this molecule is toxic or whether it is just a by-product of cellular metabolism in a pathological situation. A number of cellular proteins have been found to be nitrated in PD tissues (Ischiropoulos and al-Mehdi 1995), which was taken as evidence that nitrating agents such as peroxynitrite engaged in nitration reactions here. Specific antibodies that recognize nitrated alpha-synuclein have demonstrated that alpha-synuclein is the protein that is nitrated in Lewy bodies in a number of disease states including PD (Giasson et al. 2000a,b). Furthermore, alpha-synuclein inclusions in tissues from PD patients were strongly labeled with antibodies that recognize the faithful fingerprint of peroxynitrite-induced nitration, 3-nitrotyrosine (Souza et al. 2000). Both *in vitro* studies and the MPTP mouse model were used to prove that tyrosine residues in the alpha-synuclein molecule are indeed the targets of nitration and that peroxynitrite is indeed the culprit. In HEK 293 cells transfected to overexpress human alpha-synuclein and that were exposed to peroxynitrite, a nitrated band that corresponded to the molecular mass of alpha-synuclein was noted (Przedborski et al. 2001). In the MPTP mouse model, immunoprecipitation studies using striatum and ventral midbrain from treated mice showed that alpha-synuclein was nitrated as early as four hours after MPTP administration. In contrast, beta-synuclein was not nitrated in either situation (Przedborski et al. 2001).

Step 8. Release of Dopamine from Intracellular Stores

A second consequence of the depletion of cellular ATP is the release of dopamine from intracellular stores (Schmidt et al. 1984; Ofori and Schorderet 1987; Rollema et al. 1988; Lau et al. 1991; Schmidt et al. 1999). Once released into the extracellular space, the enzymatic oxidation of dopamine results in the rapid formation of hydroxyl radicals. It is clear that the presence of free radicals can lead to membrane damage and subsequent cell death. That dopamine rapidly auto-oxidizes and contributes to neurotoxicity always leads to the controversial topic of L-dopa therapy in PD. Simply stated, one can question whether the therapy that best treats the symptoms of PD may also exacerbate the disease. In support of this hypothesis, Whone and colleagues showed that the progression of PD using PET scanning was greater in patients treated with L-dopa than those treated with the dopamine agonist ropinirole (Whone et al. 2003). However, other studies do not support this hypothesis (Fornai et al. 2000; Melamed et al. 2000), and for this reason, the question of L-dopa toxicity has yet to be resolved.

While the above question is still not settled, the formation of hydroxyl radicals apart from direct dopamine oxida-

tion can also modulate several other processes that can lead to cell death, including the fragmentation of DNA (Walkinshaw and Waters 1995) and inhibition of Na+, K+-ATPase activity (Khan et al. 2003). Additional sites of hydroxyl radical formation may occur as a result of interactions with neuromelanin (D'Amato et al. 1986) as well as with cellular iron (Jellinger 1999), each of which could contribute to its neurotoxicity.

Step 9. A Second Role for Glial Cells?

Based upon our hypothesis of the mechanism(s) of MPTP-induced cell death (Figure 1), a dramatic interplay occurs between neurons and the non-neuronal milieu. As discussed earlier in this chapter (step 2), the astrocytes are necessary for the bioactivation of MPTP into its toxic metabolite, MPP^+. The glial cells, in addition to their toxifying function, also are believed to play a significant role in neuronal protection. A recent report, using *in vitro* chimeric cell cultures, has demonstrated that the toxicity of MPTP is determined by the response of the glial cells following MPP^+ intoxication (Smeyne et al. 2001) and numerous *in vitro* studies support this data (Di Monte et al. 1992; Forno et al. 1992; Di Monte et al. 1996).

Glial cells contribute directly to the toxic effects of MPTP through several mechanisms, including the mediation of free radical formation and damage by induction of nitric oxide synthase (iNOS) (Hirsch et al. 1998; McGeer and McGeer 1998; McNaught and Jenner 1999). Administration of MPTP leads to a rapid gliosis (Schneider and Denaro 1988), which subsequently increases production and releases iNOS (Zietlow et al. 1999). In a model of iNOS action that extends the role of glia, Hirsch and Hunot (2000) suggest that MPTP acts directly on the induction of cytokines that activates iNOS. iNOS is then released from the glial cells to directly damage the dopaminergic neurons. Thus, differential expression of iNOS may underlie some of the strain specific responses to MPTP seen in mice, and, perhaps, the differential sensitivity to different environmental toxins in humans.

In addition to inducing and modulating cytokines, dopamine in the extracellular space can induce a number of different molecules that are involved in oxidative stress. One of these molecules, hemeoxygenase-1 (Fernandez-Gonzales et al. 2000), the rate limiting enzyme in heme degradation, plays a critical role in heme and iron homeostasis (Schipper et al. 1998b; Maines 2000). Several isoforms of hemeoxygenase have been identified (reviewed in Elbirt and Bonkovsky 1999), each of which converts heme to bilirubin and carbon monoxide, while at the same time releasing iron into the cellular milieu (Maines 1997). Further support for the importance of this molecule is that hemeoxygensase-1 is elevated in astrocytes of Parkinsonian patients (Schipper et al. 1998a). In addition, the brains of hemeoxygenase-1 null mice show excessive iron deposits, increased sensitivity to oxidative stress, and chronic inflammation (Poss and Tonegawa 1997). Moreover, astrocytes in the striatum of MPTP-treated mice show increases in hemeoxygensase-1 as early as six hours after the administration of MPTP (Fernandez-Gonzales et al. 2000). On the flip-side, over-expression of hemeoxygensase-1 leads to a reduced damage in the presence of free radicals (Maines 1997) which is why investigators have postulated the induction of hemeoxygensase-1 as a potential therapy for PD. However, based on the breakdown of heme, which leads to the formation of biliverdin and carbon monoxide as well as free iron, it is possible that in the specific environment of the SNpc, hemeoxygensase-1 can act counterintuitively and lead to further neurotoxicity (Hansen 1994; Schipper 1999). The breakdown products of heme induced by hemeoxygensase-1 also may act as mitochondrial toxins, leading to a feed-forward loop that eventually leads to cell death.

In addition to participating in cellular toxicity, astrocytes, either in the substantia nigra or striatum, may also act as a protective agent through several mechanisms, including their ability to act as "cellular buffers" and by producing neurotrophic factors. Several studies show that astrocytes can aid in neuronal protection thorough the synthesis and release of the free-radical scavenger glutathione and/or its precursors glutamate, cysteine, and glycine (Drukarch et al. 1998; Dringen et al. 1999). Unlike neurons, glia can generate this neuroprotectant through the biochemical pathways that use cysteine and cystine to produce GSH (Sagara 1993 #1117; Wang and Cynader, 2000). Since GSH levels are lower in the SNpc of PD patients, the local astrocytes in the substantia nigra may serve this important function. The efficiency of glial cells in producing or in maintaining levels of glutathione in different strains of mice (Hatakeyama et al. 1996) may be an important factor in the pathogenesis of dopaminergic neuron loss in experimental models of PD and may provide a therapeutic target for neuroprotection.

In addition to providing the precursors for redox modulating compounds such as glutathione, astrocytes also produce a number of neurotrophic factors (Schaar et al. 1993, 1994; Nakajima et al. 2001). Several neurotrophins support dopaminergic neurons following MPTP or MPP^+ intoxication (Nagatsu et al. 2000). These factors include BDNF (Spina et al. 1992; Frim et al. 1994; Tsukahara et al. 1995), GDNF (Cheng et al. 1998; Date et al. 1998), FGF (Otto and Unsicker 1994), and EGF (Hadjiconstantinou et al. 1991). Neurotrophins act to prevent cell death through a number of mechanisms including interference with the intrinsic cell death programs (Schabitz et al. 2000; Heaton et al. 2003) and modulating oxidative stress (Spina et al. 1992; Kirschner et al. 1996; Skaper et al. 1998; Gong et al. 1999; Petersen et al. 2001).

III. CONCLUSIONS

The discovery that MPTP, which is structurally similar to a number of commonly used herbicides and pesticides, can induce specific loss of substantia nigra neurons in many vertebrate species, from humans to mice, has lead to the development of a useful model of Parkinson disease. In mice, MPTP demonstrates differential toxicity that is dependent on the strain of animal examined (Sonsalla and Heikkila 1988; Muthane et al. 1994; Hamre et al. 1999). This finding supports the hypothesis that the loss of substantia nigra neurons in Parkinson disease may result from a genetic sensitivity to a number of environmental agents (Veldman et al. 1998; Stoessl 1999). In a recent study, the chromosomal loci containing the genetic sequences responsible for this sensitivity was identified on the telomeric end of mChr.1 (Cook et al. 2003). Further studies into the genetic and biochemical pathways involved in MPTP toxicity will lead to a better understanding of idiopathic Parkinson disease and provide clues to novel targets for therapeutic interventions.

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Review

The MPTP model of Parkinson's disease

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Abstract

The biochemical and cellular changes that occur following administration of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) are remarkably similar to that seen in idiopathic Parkinson's disease (PD). In this review, we detail the molecular activities of this compound from peripheral intoxication through its various biotransformations. In addition, we detail the interplay that occurs between the different cellular compartments (neurons and glia) that eventually consort to kill substantia nigra pars compacta (SNpc) neurons.

Keywords: Parkinson's disease; Glial cells; Substantia nigra; MPTP

Contents

1. Introduction

Parkinson's disease (PD) was first described in a paper entitled "An Essay on the Shaking Palsy" in 1817 by Dr. James Parkinson in Ref. [109]. PD is a progressive neurological disorder that strikes 1–2% of the "over 50" population [21]. Current estimates from the American Parkinson's Disease Foundation put the number of American citizens suffering from this disease at greater than 1.5 million persons. At this time, PD is the third most prevalent neurodegenerative disorder, following Alzheimer's disease and dementia with Lewy body disease. Since the disease incidence increases with age, it is likely that the number of people suffering from PD will rise as improved healthcare lengthens the average life span.

The main anatomical feature of PD is the decrease in number of neuromelanin-containing neurons located in the midbrain substantia nigra pars compacta (SNpc). These dopaminergic neurons project to the striatum as well as a number of other subcortical regions [161]. PD symptoms first manifest when approximately 60% of the SNpc neurons have already died [39] and 70% of dopamine responsiveness disappears [83]. Because the progression of cell loss is thought to occur over a somewhat protracted period of time in a defined spatiotemporal manner [18,104],

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the onset of Parkinson's disease symptoms is often insidious.

At this time, it is unclear as to how much of the disease results from a purely environmental factor, a strict genetic causation, or a combination of the two [23,146]. Most epidemiological studies conclude that less than 10% of PD has a strict familial etiology [110]. This includes a small number of familial parkinsonian patients with polymorphisms in the α-synuclein gene [112] (reviewed by Lundvig et al., this issue) as well as patients with early onset PD that have recessive mutations in the Parkin gene, mapping to the long arm of chromosome 6 (6q25.2-q27). The pathogenicity of these proteins is discussed in a review by Burke [9]. Familial PD has also been associated with human chromosome 2p13 and 4p polymorphisms [38]. The PD linked to this locus more closely resembles that of idiopathic PD, although like the α-synuclein protein, this unknown protein has very low penetrance. Although alterations in the proteins coded for by these loci may lead to an understanding of the molecular processes that occur in idiopathic PD, no mutations as of yet have been reported in aged-onset idiopathic PD [56,138].

Since the majority of PD patients have no identifiable genetic mutation, important information regarding the pathophysiology of PD may be learned through the study of animal models. At this time, several animal models have been developed to study the underlying mechanisms that lead to the development of experimental PD. One of the earliest models made use of a lesion of nigrostriatal pathway in which fibers emanating from the substantia nigra proceeded to the striatum rostrally through the medial forebrain bundle, [6,31,80]. Other models have used chemical lesions. One example is the use of 6-OHDA, a neurotoxin that when injected into the striatum causes a retrograde degeneration of dopaminergic neurons in the SNpc (reviewed in Refs. [22,55,107,137]). Another model of experimental PD utilizes the properties of selective neurotoxins, including 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP).

The sequalae of degeneration that occurs following administration of MPTP in animals has provided a useful model of Parkinsonism because it induces pathologies similar to that seen in man. Interestingly, the use of MPTP may be one of the few cases in which the effects of a neurotoxin were discovered in humans first, followed by development of an animal model. Although there were early reports of neurotoxicity to this compound, its use as a research tool became prevalent in the early 1980s following the identification of a number of Northern California heroin users who presented at various emergency rooms with symptoms indistinguishable from those of Parkinson's disease [10,75]. A complete history of these cases is presented in "The Case of the Frozen Addicts" [76].

Although MPTP was first identified as a parkinsonian agent in humans, it has been demonstrated to exert similar effects in a number of other primates [63,70,157], as well as in cats, and in several rodents. In rodents, it has been shown that only specific strains of mice are sensitive to the administration of MPTP [46,94,123,148]. MPTP structurally resembles a number of known environmental agents, including well-known herbicides, such as paraquat [24], and garden insecticides/fish toxins, such as rotenone [90], that have been shown to induce dopamine cell degeneration [4,8,13,153], although mechanistically, the actions of each are likely different [24]. Further studies of each of mechanism of each of these toxins may lead to a unified pathway that underlies these toxins.

2. Mechanism of MPTP action

There are many points systemically where MPTP can affect the dopaminergic system (Fig. 1). The first point of potential modulation of any exogenous compounds neurotoxicity is the blood–brain barrier (BBB). The BBB is composed of tight-junctioned endothial cells that make up the microvasculature of the brain in tight opposition with the end feet of glial processes. MPTP first is metabolized by the enzyme MAO-B to 1-methyl-4-phenyl-2, 3-dihydropyridium ($MPDP^+$) that then deprotonates to generate the corresponding pyridium species, MPP^+. Endothelial cells in the microvasculature that make up the BBB contain monoamine oxidases; and several studies have correlated levels of monoamine oxidases with MPTP-induced neuronal loss [64,124]. Since MPP^+ cannot be transported through the BBB [125], this level of toxification/detoxification can provide a first line of defense against exogenous agents.

3. Role of glial cells (part 1)

MPTP that is not converted to MPP^+ in the periphery rapidly enters the brain where it is processed into glial cells by a number of mechanisms, including monoamine [7] and glutamate [50] transporters or pH-dependent antiporters [69,87]. Glia, like the previously mentioned endothelial cells, also contain large pools of monoamine oxidases, and also convert MPTP from its protoxin form to MPP^+ [122]. Additional support for the role of glial cells in dopaminergic neuronal toxicity was shown by Brooks et al. [7] who demonstrated that administration of a serotonergic uptake inhibitor, fluoxetine, immediately before systemic injection of MPTP altered the observed neurotoxicity. Since fluoxetine did not alter the neurotoxicity of injected MPTP, it was proven that the site of activation was extraneuronal, lending credence to the observation that the primary step in MPTP toxicity occurred in the astrocyte.

Once converted to MPP^+ in the astrocyte, MPP^+ stimulates the up-regulation of TNF-α, interleukin-1β (IL-1β) and IL-6 [152,160] and these, in turn, up-regulate inducible nitric oxide synthase (iNOS) [57]. Of the three NOS isoforms present in the brain, endothelial NOS

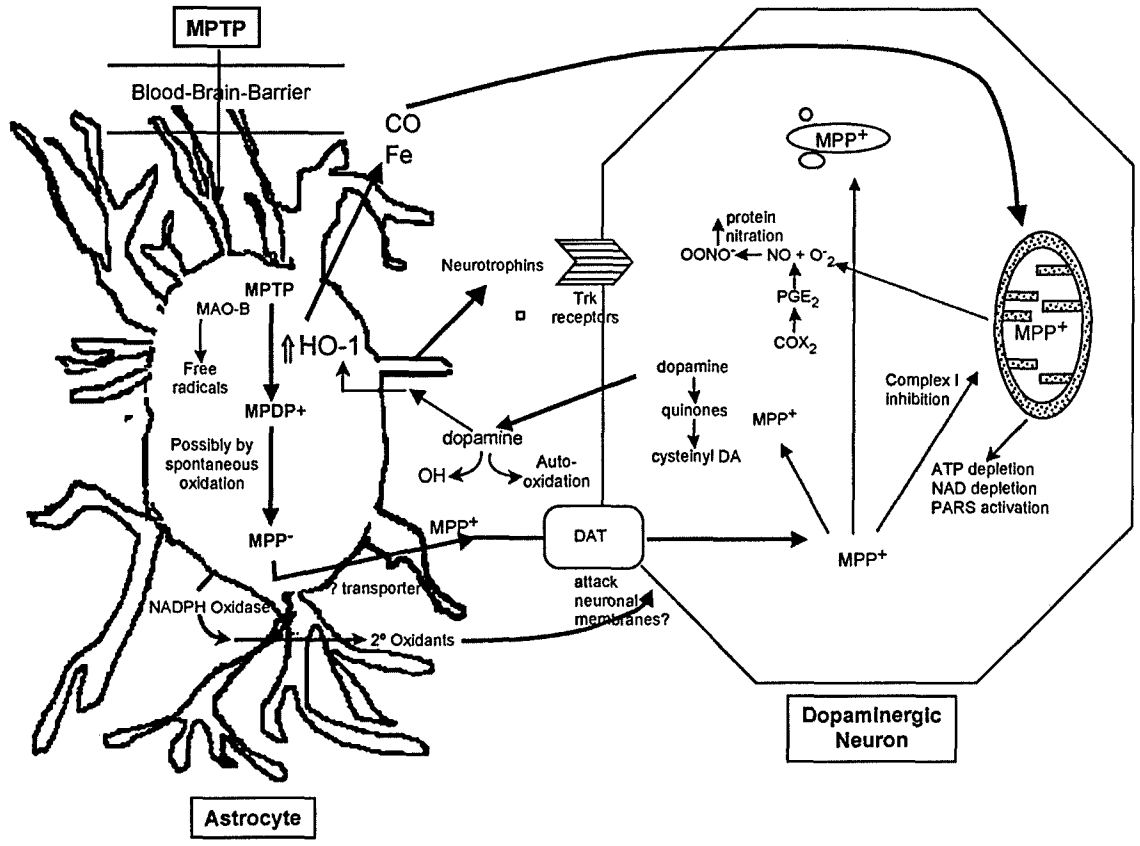

Fig. 1. Schematic representation of the mechanism of MPTP action in the nigrostriatal system. Red arrows represent the initial (toxification) role of glial cells. Blue arrows represent the second (neuroprotective) role of glial cells.

(eNOS), found mainly in the vasculature of the brain, is not altered following to MPTP toxicity [98]. In addition, since neuronal NOS (nNOS) knockout mice show partial protection against the MPTP toxicity in the substantia nigra pars compacta (SNpc) [116], another NOS isoform must also contribute to the neurotoxicity of MPTP. iNOS, a NOS isoform that is minimally expressed in the normal brain, has been shown to be up-regulated in the substantia nigra's microglia in both Parkinson's disease (PD) and in mice following MPTP treatment [68,81,158,159]. iNOS produces large amounts of the uncharged and lipophillic molecule nitric oxide (NO) and as such can freely pass through membranes and travel distances greater than the length of a neuron [73]. Thus, following MPTP treatment, neurons in the vicinity of the NO molecule are put at risk for possible attack by glial-derived reactive nitrogen-related species. Since iNOS induction can be blocked by the antibiotic minocycline [158], this step in the toxification process of MPTP presents a point where potential therapeutics may have a significant impact.

Since MPP^+ is a polar compound, it cannot freely exit from its glial environs. It has been suggested that there may be a specific transporter that actively moves this polar molecule out of the glia [58,127]; however, at present, this specific mechanism remains unknown.

Once MPP^+ is released into the extracellular space, MPP^+ is taken up into dopaminergic cells by the dopamine transporter (DAT). Since midbrain neurons contain the highest concentration of dopamine transporters/cell [11], the DAT may be a control point in determining how susceptible midbrain neurons are to exogenous agents [67,79] (but see also Ref. [52]). The requirement for the DAT in relation to MPTP toxicity was demonstrated by two groups examining mice carrying null mutations of the DAT [5,36]. In these studies, MPTP-susceptible strains of mice carrying null mutations of the DAT were completely protected from MPTP toxicity.

4. Role of dopaminergic neurons

Once in the cell, MPP^+ can move through several cellular compartments: it can enter into mitochondria where it interferes with complex I of the electron transport chain [74,103] or it can be sequestered into cytoplasmic vesicles by actions of the vesicular monoamine transporter [20,82].

MPP^+ enters the mitochondria by the diffusion through the mitochondrial inner membrane. The uptake of MPP^+ into mitochondria is actively driven by a membrane electrical gradient ($Km \approx 5$ mM). This active transport was supported by experiments in which valinomycin plus potassium, which collapses the mitochondrial electrochemical gradient, eliminated MPP^+ uptake, while agents which

collapsed this proton gradient had no effect on MPP^+ uptake [120,121].

Once in the mitochondria, MPP^+ inhibits cellular respiration through the blockade of the electron transport enzyme NADH:ubiquinone oxidoreductase (complex I) [102,149]. Blockade of this complex leads to a reduction in cellular ATP. Although this appears to be the major step in blockade of mitochondrial function, studies have shown that MPP^+ can also directly inhibit complexes III (ubiquinol:ferrocytochrome c oxidoreductase) and IV (ferrocytochrome *c*:oxygen oxidoreductase or cytochrome *c* oxidase) of the electron transport chain [95,96]. The loss of cellular energy through each of these pathways has several consequences, including the generation of the oxygen free radicals that rearranges to form hydrogen peroxide. Further catalysis leads to the formation of hydroxyl radicals.

Based upon the finding that MPP^+ depletes cellular energy due to interference with complex I–III, and as such may be related to the etiology of human PD, a number of potential therapies have been examined. One promising study has used Coenzyme Q10 supplementation, where oral administration of this compound in fairly high doses has been observed to slow the progression of the disease [2,97,139].

Although complex I inhibition by MPP^+ reduces energy production within dopaminergic neurons, it is likely that this is not the immediate cause of the SNpc neuronal death. The damage done within these dopaminergic neurons is likely to result from compounds generated in the cell, secondary to energy depletion. The formation of the superoxide radical is one example of this process. Cleeter et al. [14] showed that MPP^+, following inhibition of mitochondrial complex 1 activity, formed an excessive amount of superoxide radicals within the neuronal cytosol. Further support for the role of superoxide radicals came from Przedborski et al. [115], who demonstrated that overexpression of the copper–zinc form of superoxide dismutase in mice is neuroprotective against the damaging effects of MPTP. Moreover, Wu et al. [159], using the fluorescent tag hydroethidium, provided an in vivo demonstration of the presence of the superoxide radicals following MPTP intoxication.

NO, produced and released by glial cells, can enter the cytosol of the neuron via simple membrane diffusion. At this point, the superoxide radical and NO, which are not particularly damaging by themselves; can interact to form peroxynitrite ($OONO^-$), one of the most destructive oxidizing molecules [59,114,117]. Although difficult to detect due to its rapid processing, the nitration of the tyrosine residues of a number of cellular components that include enzymes, transmitters, proteins, fatty acids and DNA can easily be identified [119].

One potential target of $OONO^-$ is tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. The most densely packed TH-positive cell area in the brain is the SNpc, which projects fibers to the striatum [44]. Since it has been shown that the cell-body rich SNpc primarily contains the soluble form of the TH enzyme, it is often used as the phenotypic marker for dopaminergic neuron numbers and can be measured both by biochemical and immunohistochemical methods to determine neuron loss [46,60].

Clinical symptoms are first thought to appear when about 60–70% of the TH-positive cells in the SNpc have degenerated [30]. Brains of parkinsonian patients also show deficits in TH enzyme and TH enzyme activity [61]. Both in vitro and in vivo studies demonstrate that peroxynitrite impairs TH activity [71]. In MPTP-treated mice, TH nitration occurs soon after MPTP administration. Furthermore, transgenic mice that overexpress human SOD do not show any detectable levels of nitrated striatal TH following MPTP treatment [1]. Mice deficient in iNOS show less ventral midbrain nitrotyrosine, a fingerprint for tyrosine nitration, after MPTP administration than in their wild-type counterparts [81]. Thus, the inactivation of TH via its nitration following exposure to both peroxynitrite and MPTP appears to be an important process in the development of PD in humans and to the MPTP neurotoxic process in mice.

Dopamine (DA) is a relatively unstable molecule that is subject to both hydroxyl radical attack [141] and autooxidation in the extracellular space [53]. In addition to extraneuronal effects, dopamine can also be nitrated intracellularly [78] and therefore may contribute to the degeneration of the cells that contain this neurotransmitter. In this process, DA is oxidized to DA quinone, which then undergoes a nucleophilic addition via the transfer of a sulfhydryl group from cysteine, to form 5-cystenyl-DA [43]. In pathological situations, the oxidation of DA to 5-cystenyl-DA is facilitated by the up-regulation of cyclooxygenase-2 (COX-2) [48,105]. The role of 5-cystenyl-DA in the development of PD as well as to the degeneration of DA neurons seen in the MPTP mouse model was examined in a variety of studies. For example, it was observed that COX-2 immunostaining was robust in the human and mouse dopaminergic neurons [151]. Additionally, COX-2 enzyme activity and protein levels in both PD brains and ventral midbrain from MPTP-treated mice were found to be significantly higher than in controls. Inhibition of the COX-2 response to MPTP, however, prevented the rise in protein cysteinyl dopamine that was seen to occur in mice following the administration of MPTP [151].

The vesicular monoamine transporter VMAT2, is a proton-dependent transporter that sequesters monoamine neurotransmitters from free cytoplasmic space into synaptic vesicles [93]. Since it structurally resembles monoamines, MPP^+ can be transported by the VMAT into these vesicles, thus being prevented from entering the mitochondria where it can inhibit complex I. This sequestration has been hypothesized to be as a potential mechanism for reducing the deleterious effects of any number of monoaminergic toxins. Support for this hypothesis comes from analyses in mice containing partial or complete deletions of VMAT2 as well as from human studies of VMAT expression. In parkinsonian humans, immunocytochemical localization of

VMAT showed reduced expression in striatum, similar to that seen in the DAT. In fact, Miller et al. [92] suggested that the relative expression of VMAT2, compared to that of DAT, may allow one to predict if and which dopamine neurons may be lost in PD (see the possible application of this hypothesis in Faherty et al., this issue). In animal studies, VMAT2(±) mice exposed to MPTP were examined for markers of dopaminergic neuron toxicity, including dopamine content and DAT protein in the stiatum, as well as expression of glial fibrillary acidic protein (GFAP) mRNA. In all parameters measured, VMAT2(±) mice were more sensitive than their wild-type littermates to MPTP-induced toxicity [37]. Further examination of these mice revealed that VMAT2(±) mice, following administration of MPTP, also had increased SNpc cell loss [150]. These studies suggested an important role for VMAT2 in potentiating the effects of MPTP. Using an in vitro system, cells transfected to overexpress a greater density of VMAT2 were converted from MPP^+-sensitive to MPP^+-resistant cells [82]. These studies suggested an important role for VMAT2 in modulating the effects of MPTP. Another molecule of interest relevant to the development of PD in humans and to the neurotoxic process in the MPTP mouse model of PD is α-synuclein. Synucleins are cytosolic proteins that contain 127–140 residues that have a unique 11-residue repeat that occurs in 5–7 copies which accounts for roughly one-half of their structure and no structural domains (see review by Lundvig et al., this issue). Four proteins, α, β and γ synuclein as well as synoretin make up this family of proteins of which only two, α and β, are synthesized in relatively large amounts in the brain (making up approximately 1% of total brain protein). These proteins are generally found in abundance in presynaptic nerve terminals [134]. Mutations in α-synuclein have been associated with a familial form of PD [112] that is readily indistinguishable from the more common sporadic form of the disease. It is thought that the interaction between WT and mutant α-synucleins may enhance the ability of these proteins to interact with other non-synuclein cellular proteins to form aggregates [15].

The presence of Lewy bodies within neurons in the SNpc is one of the characteristic pathologies seen in PD. Lewy bodies are both ubiquitin and α-synuclein immunopositive. Since α-synuclein is the only synuclein present in Lewy bodies, it has to be determined whether this molecule is toxic or whether it is just a by-product (tombstone) of cellular metabolism in a pathological situation. A number of cellular proteins have been found to be nitrated in PD brains [59] and specific antibodies that recognize nitrated α-synuclein have been used to demonstrate that alpha-synuclein is the protein that is nitrated in Lewy bodies [40,41]. Furthermore, α-synuclein inclusions in tissues from PD patients have been shown to be strongly labeled with antibodies that recognize the hallmark of peroxynitrite-induced nitration, 3-nitrotyrosine [144]. Two lines of evidence support these conclusions. In vitro studies using HEK 293 cells transfected to overexpress human α-synuclein that were exposed to peroxynitrite showed a nitrated band that corresponded to the molecular mass of α-synuclein was noted [118]. In vivo, using the MPTP mouse model, immunoprecipitation studies of midbrain and striatum showed that α-synuclein was nitrated as early as 4 h after MPTP administration. Specificity for this form of synuclein was demonstrated by the observation that β-synuclein was not nitrated in either situation [118].

Another consequence of the cellular ATP depletion is the abnormal release of DA from intracellular stores [77,106,126,135,136]. Once DA is released into the extracellular space, the enzymatic oxidation of DA results in the formation of hydroxyl radicals. That dopamine rapidly auto-oxidizes and contributes to neurotoxicity always leads to the controversial topic of L-dopa therapy in PD. Simply stated, one can question whether the therapy that best treats the symptoms of PD may also exacerbate the disease. In support of this hypothesis, Whone et al. showed that the progression of PD using PET scanning was greater in patients treated with L-dopa than those treated with the dopamine agonist ropinerole [86,156]. However, other studies do not support this hypothesis [33,91], and for this reason, this question has yet to be sorted out.

Additional sites of hydroxyl radical formation may occur as a result of interactions with neuromelanin [17] as well as with cellular iron [62], each of which could contribute to its neurotoxicity.

5. Role of glial cells (part 2)

The mechanism(s) of MPTP-induced cell death (Fig. 1) show a great amount of cross-talk between the neurons and the nonneuronal milieu. Previously, we discussed how the astrocytes are necessary for the conversion of MPTP to MPP^+. In addition to this function, astrocytes are also believed to play a significant role in neuroprotection. A study using chimeric SN cell cultures, has demonstrated that the differential toxicity of MPTP in mouse strains is determined by the response of the glial cells [142]. This work is supported by other in vitro studies [25,26,34].

As discussed earlier in this review, glial cells directly contribute to the toxicity seen following administration of MPTP through several mechanisms, including the mediation of free radical formation and damage by induction of nitric oxide synthase (iNOS) [54,88,89]. In addition to the induction and modulation of cytokines, the presence of dopamine in the extracellular space can induce a number of different molecules that are involved in oxidative stress. One of these molecules, hemeoxygenase-1 (HO-1), the rate-limiting enzymes in heme degradation, has been shown to play a critical role in iron and heme homeostasis [85,133]. It is well known that alterations in brain iron are seen in PD brains [3,65,72]. Several isoforms of hemeoxygensase have been identified (reviewed in Ref. [29]), each of which converts

heme to bilirubin and carbon monoxide, while at the same time releasing iron into the cellular milieu [84]. Further support for the importance HO-1 is that it is elevated in astrocytes of parkinsonian patients [132]. In striatal astrocytes, HO-1 elevation occurs as early as 6 h following administration of MPTP [32]. In addition, brains of HO-1 null mice show excessive deposition of iron, increased sensitivity to oxidative stress and chronic inflammation [113]. On the flip side, overexpression of HO-1 leads to a lessening of damage that has been observed in the presence of free radicals [84]. For this reason, modulation of HO-1 has been postulated as a potential therapy for PD. However, based on the breakdown of heme, which leads to the formation of bilivirdin, carbon monoxide and free iron, it is possible that in the specific environment of the SN, HO-1 can act counterintuitively and lead to a furthering of neurotoxicity [47,131]. It is also possible that the breakdown products of heme induced by HO-1 act as mitochondrial toxins leading to a feed-forward loop that eventually leads to cell death.

In addition to acting as participants in cellular toxicity, astrocytes, either in the substantia nigra or striatum, may also act as a protective agent through several mechanisms, including their ability to act as a "cellular buffers" and production of neurotrophic factors. Several studies have shown that astrocytes synthesis and release the free-radical scavenger glutathione and/or its precursors glutamate, cysteine and glycine [27]. This function is specific to astrocytes and not neurons—as they are able to generate this neuroprotectant thorough the biochemical pathways that use cystine as well as cysteine for the production of GSH (Sagara, 1993 #1117, [28,155]). Since GSH levels are lower in the SNpc of PD patients, the local SNpc astrocytes may serve this critical function. The efficiency of glial cells in producing or in maintaining levels of glutathione in different strains of mice [49] may be an important factor in the pathogenesis of dopaminergic neuron loss in experimental models of PD and may point to this pathway as a therapeutic target for neuroprotection.

In addition to providing the precursors for redox-modulating compounds, such as glutathione, astrocytes have also been shown to produce a number of neurotrophic factors [101,128,129]. Several neurotrophins have been shown to protect dopaminergic neurons from cell death following MPTP or MPP^+ intoxication [100]. These factors include BDNF [35,145,154], GDNF [12,19], FGF [108] and EGF [45]. Neurotrophins act to prevent cell death through a number of mechanisms including modulation of oxidative stress [42,66,111,140,145] as well as interference with the intrinsic cell death programs [51,130].

6. Conclusions

MPTP, which is structurally similar to a number of commonly used herbicides and pesticides, can induce specific loss of substantia nigra neurons in many vertebrate species, from man to mouse. Studies using this toxin have led to the development of useful animal models of Parkinson's disease. In mice, MPTP demonstrates differential toxicity that is dependent on the strain of animal examined [46,99,143] (see also Pierri et al. in this issue). This finding supports the hypothesis that the loss of substantia nigra neurons in Parkinson's disease may result from a genetic sensitivity to a number of environmental agents [24,147]. Recently, the chromosomal loci containing the genetic sequences contributing to this sensitivity to neuronal loss [16] in mice has been localized on the telomeric end of mChr.1 [16]. Further studies into the genetic as well as into the biochemical pathways involved in MPTP toxicity will lead to a better understanding of idiopathic Parkinson's disease as well as provide clues to novel targets for therapeutic interventions.

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APPENDIX 9

Minireview

MPTP and the SNpc DA Neuronal Environment : Role of Dopamine, Superoxide and Nitric Oxide in Neurotoxicity

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Abstract.

Parkinson disease is a common neurodegenerative disease of unknown origin that is characterized, mainly, by a significant reduction in the number of dopamine neurons in the substantia nigra pars compacta of the brain. For reasons that we do not know, the dopamine neuron seems to be more vulnerable to damage than any other neuron in this area of the brain. Although hypotheses of damage to these neurons include oxidative stress, growth factor decline, excitotoxicity, neuroinflammation and protein processing dysfunction, neuroinflammation garners a significant amount of interest. This means that the survival and function of the dopamine neuron is highly dependent on its external and internal environments. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) has been used extensively to sort out what happens in the environment in and around the dopamine neuron. Superoxide, nitric oxide and dopamine are the main contributors here and it seems that the external and internal environments of the dopamine neuron are similar in PD and in the MPTP mouse model of PD.

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability (Fahn and Przedborski, 2000), these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fahn and Przedborski, 2000). There are, at present, 1 million PD patients in the United States alone, with 50,000 newly diagnosed cases each year (Fahn and Przedborski, 2000). These cases include both familial and sporadic PD, of which sporadic PD appears to be the more common (Dauer and Przedborski, 2003). Currently, the most effective therapy for alleviating the symptoms of PD is levodopa (L-DOPA) (Fahn and Przedborski, 2000), which increases the levels of dopamine in the brain, but does not seem to alter the progression of the disease. Furthermore, speculations exist that levodopa may actually contribute to the progression of PD (Fahn, 1997; Weiner, 2000). For reasons that are not yet understood, dopaminergic neurons in the SNpc appear to be more susceptible to damage than other neurons in the brain. Theories as to why this situation exists include oxidative stress (Fahn and Cohen, 1992; Przedborski and Jackson-Lewis, 2000), growth factor decline (Mogi et al, 1999; Nagatsu et al, 2000), excititoxicity (Olanow and Tatton, 1999), neuroinflammation due to changes in the neuronal environment (Langston et al, 1999; Hunot and Hirsch, 2003, Teismann et al, 2003) and, more recently, protein processing dysfunction (Ii et al, 1997; Trojanowski et al, 1998). Progression of a number of neurological diseases is related to inflammation in the brain which affects the neuronal environment. For

instance, multiple sclerosis is a neuroinflammatory disease that causes a loss of the myelinated tracts in the CNS wasting away of muscle fibres (Hafler, 2004) and recent evidence has shown that there is an inflammatory component to amyotrophic lateral sclerosis (Drachman et al, 2002). Furthermore, supporting a role for inflammation in the neuronal environment in PD is the finding by Langston and colleagues that brains from individuals, who died from a PD-like syndrome resulting from the self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and who lived for 3-16 years following exposure, showed a significant presence of activated microglia (Langston et al, 1999). Thus, it is conceivable that the progression of PD may be assisted by changes in the external and internal environments in and around the SNpc DA neuron which promote neuronal death rather than support life.

PD and the Neuronal Environment.

As mentioned above, one of the biggest hints to this possibility that inflammation in the neuronal environment may be a part of the progressive nature of PD rests with Langston's finding of activated microglia in brains from humans who injested MPTP. However, in addition to Langston's discovery, a number of suggestions were already in evidence. For example, one of the earliest indicators of the presence of an environmental imbalance in the brain was the up-regulation of major histocompatability complex II (MHC class II) positive microglia in the SNpc and of MHC I in the striatum of postmortem PD brains (McGeer et al, 1988; Mogi et al, 1995). The role of each complex is different, thus the question

remains as to whether the up-regulation of both MHC complexes has some functional significance in neurodegeneration in the nigrostratal pathway or simply denotes the activated status of the microglia (Hunot and Hirsch, 2003). Increases in cytokines, both pro- and anti-inflammatory, found in the nigrostriatal pathway (Nagatsu et al, 2000) as well as systemically (Bessler et al, 1999) in PD patients are also indicative that some kind of environmental change has taken place. These include, but are not limited to: a) proinflammatory cytokines such as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interferon N-gamma (IFN-γ); b) the anti-inflammatory cytokine interleukin-4 (IL-4); and c) growth factors such as TGF-α, TGF-β1, EGF and bFGF (Bessler et al, 1999; Nagatsu et al, 2000; Hunot and Hirsch, 2003). The roles of these agents in the PD scenario, however, still remain to be clarified. Furthermore, one of the biggest pieces of the PD puzzle has been the direct demonstration of the presence of the inducible form of nitric oxide synthase (iNOS) in glial cells from PD brains. Hunot et al (1999) showed not only the presence of iNOS protein expression in human glial cells but also connected this iNOS up-regulation to activation of the cytokine CD23. The fact that other investigators have shown that the density of glial cells expressing iNOS is greatly increased in the SNpc from PD patients (Hunot et al, 1996), that nitrites in cerebrospinal fluid from PD patients are increased (Qureshi et al, 1995), and that 3-nitrotyrosine (evidence of peroxynitrite interactions) was detected in the SNpc of PD brains (Good et al, 1998), all point to iNOS involvement in neuroinflammation. Thus, there are a lot of pieces to the inflammation puzzle and now it remains for us to put these pieces into some kind

of sequence of events, in order to discern the involvement of changes in the neuronal environment in the progression of PD. Our efforts to make some sense out of the neuroinflammation story comes through the use of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). We and others have used MPTP to follow the neuroenvironmental change hypothesis, and findings using this compound have mimicked what has been found in PD brains thus far.

MPTP Mimics the DA Neuronal Environment in PD.

The non-neuronal support system in the CNS are the glial cells (Abbott, 1988). Under physiological conditions, glia secrete substances into the extracellular environment that support the normal functioning of the neuron (NJ Abbott, 1988). As stated above, the DA neuron in the SNpc seems to be more susceptible to damage with respect to other neuron in this area of the brain but the reason for this susceptibility remains unknown. MPTP, the tool of choice for investigations into the etiology and sequence of events in PD, has really opened the door on an inflammatory process that may also be a hallmark of the disease. Basically, this compound kills dopaminergic neurons in a time-dependent manner (Jackson-Lewis et al, 1995) and even though the MPTP-induced DA neuron death is reminiscent of "end-stage" PD (Przedborski et al, 2000), the cascade of events that MPTP initiates is key to our understanding of the sensitivity of and the death of dopaminergic neurons in the SNpc in PD. MPP^+, the toxic metabolite of MPTP, is an early clue to the changes in the DA neuronal environment. Taken up into astrocytes (Brooks et al, 1989; Kopin, 1992; Hazell et al, 1997), MPTP is

metabolized to MPP^+ by monoamine oxidase-B (Ransom et al, 1987). Before it can injure the DA neuron directly, MPP^+ must first exit the glia in which it is produced by a mechanism that thus far eludes us. Once in the extracellular space, MPP^+ enters the DA neuron through the DA transporter (Kostic et al, 1996), where it produces an oxidative stress situation (Przedborski and Jackson-Lewis, 1998) within the neuron through the stimulation of the superoxide radical (Przedborski et al, 1992; Wu et al, 2003), nitric oxide (NO) (Przedborski et al, 1996; Liberatore et al, 1999) and intracellular DA oxidation (Lotharius and O'Malley, 2000). Thus, the neuronal environment can either be supportive of or destructive of the DA the neuron.

MPTP, Superoxide and the SNpc Extraneuronal Environment.

The environment surrounding SNpc neurons can control the fate of these cells. For example, following MPTP administration, both the extracellular and the intracellular environments of the SNpc DA neuron are altered in such a way that they are no longer part of a supportive system but rather contain detrimental components. Our early studies using transgenic mice that overexpress the copper-zinc form of superoxide dismutase (CuZnSOD) and that were treated with MPTP show that the SNpc of these mice was protected against the damaging effects of MPTP (Przedborski et al, 1992), thus implying the involvement of the superoxide radical. Furthermore, Wu et al (2003) has shown that the infusion of SOD1 into the striatum of MPTP-treated mice is neuroprotective to SNpc neurons, thus defining a role for the superoxide radical in the MPTP neurotoxic process. Since

CuZnSOD is an extracellular enzyme (Fridovich, 1995), these results suggest that the extracellular environment of the DA neuron is perturbed or altered by the superoxide radical early in the neurotoxic process.

A significant source of the superoxide radical in the extracellular environment is NADPH oxidase (Wu et al, 2003; Gao et al, 2003). NADPH oxidase is a multimeric microglial enzyme that is composed of a number of subunits that include $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p40^{phox}$ (Babior, 1999). In resting microglia, this enzyme is inactive because $gp91^{phox}$ and $p22^{phox}$ are separated from the other phox subunits. However, following MPTP administration to mice, the NADPH oxidase complex within the microglia becomes activated because the p47 phox subunit is phosphorylated followed by the movement of the whole complex to the microglial membrane where it assembles with $gp91^{phox}$ and $p22^{phox}$. This makes the NADPH oxidase complex able to stimulate the production of the superoxide radical. Wu et al (2003), using hydroethidium injections to MPTP-treated mice, visualized the presence of the superoxide radical within microglia located in the SNpc environment of these mice. Up-regulation of NADPH oxidase in postmortem SNpc tissues from PD brains was also shown (Wu et al, 2003). The superoxide radical is then extruded into the extracellular environment where its presence not only alters the neuronal environment but also stimulates the production of secondary oxidants (Babior, 1999) which can, in turn, influence the integrity of the DA neuronal membrane, enter the DA neuron and change its internal environment.

MPTP, Nitric oxide and the SNpc Extracellular Environment.

Nitrative stress related to NO has been documented in PD brains through demonstration of the presence of the inducible form of nitric oxide synthase (iNOS) (Hunot et al, 1996; Hunot et al, 1999) and has been tied, in part, to the activated glia in the vicinity of SNpc DA neurons. Evidence of the up-regulation of iNOS in glia following MPTP administration points to an indirect process rather than a direct up-regulation of this enzyme (Ciesielska et al, 2003). In glia within in the area of the SNpc and the striatum, MPP+ stimulates the up-regulation of proinflammatory cytokines such as TNF-α, interleukin-1-Beta (IL-1β) and interleukin-6 (IL-6) (Youdim et al 2002; Teismann et al, 2003) in a time-dependent manner (Hebert et al, 2003) as early as 12-18 hours prior to the induction of iNOS (Hunot et al, 1999). Immunohistochemical studies (Liberatore et al, 1999; Dehmer et al, 2000) show that iNOS up-regulation occurs in microglia 24 hours after the administration of MPTP which suggests that the proinflammatory cytokines may stimulate the up-regulation of the iNOS enzyme and thereby increase the production of NO within the glia. In a personal communication, Wu and Przedborski noted that endothelial NOS (eNOS) is found in the brain vasculature and does not contribute to the MPTP neurotoxic process. Furthermore, neuronal NOS (nNOS), shown to be decreased within non-DA neurons in the SNpc following MPTP administration (Watanabe et al, 2004), probably contributes to the alterations in the intraneuronal rather than the extraneuronal SNpc space because of its location. Moreover, since nNOS

knockout mice were partially protected against the damaging effects of MPTP (Przedborski et al, 1996), it is likely nNOS is not the sole contributer to NO production and that another NOS isoform, of which there are three, contributes to the extracellular increases in NO production and to the alterations in the extraneuronal environment of the DA neurons in the SNpc.

NO is not a free radical, is highly lipophilic, can readily traverse membranes without the need of a transport system and has the ability to travel as far as 300 microns from its site of production (Lancaster, 1996). Under physiological conditions, both nNOS and iNOS produce significant amounts of NO that are ever present in the extracellular space while levels of the superoxide radical, constantly produced in many biological reactions within the brain, are kept in check by the abundance of SOD. In the pathology of PD and in the MPTP model, increased amounts of the superoxide radical and NO are pushed into the extracellular milieu surrounding the DA neuron. Here, they can react with each other at a faster rate than the superoxide radical can be dismutated by the extracellular CuZnSOD to produce the most damaging secondary oxidant peroxynitrite (Przedborski et al, 2000). Peroxynitrite can damage neuronal membrane proteins and lipids (Przedborski et al, 2000). Thus, the extracellular neuronal environment of SNpc is disturbed or compromised and is no longer supportive for the DA neuron either in PD or in the MPTP model.

DA and the External SNpc Environment.

The DA neuron in the SNpc may indeed be, at least in part, a contributor to its own death. Following MPTP administration, huge amounts of DA are released from intracellular stores into the extracellular space (Lau et al, 1991; Schmidt et al, 1999). Once released, DA is either enzymatically metabolized by monamine oxidase-B to 3,4-dihydroxyphenylacetic acid and in the process, the hydroxyl radical is kicked out (Burke et al, 2004) or it auto-oxidizes to form a number of toxic compounds among which is 6-hydroxydopamine (Graham, 1978) .6-hydroxydopamine is a known neurotoxin that has been used extensively for animal models in PD research (Jeon et al, 1995; Przedborski et al, 1995). It has been demonstrated that this compound destroys striatal DA terminals which die back causing the death of the DA neuron in the SNpc (Przedborski et al, 1995). Interestingly, one of the findings in PD and in the MPTP model is that there is a greater loss of DA nerve terminals than DA cell bodies in the SNpc (Fahn and Przedborski, 2000). Although 6-hydroxydopamine has never been measured in brain tissues from PD patients nor in brains from the MPTP model, one can speculate on the possibility that 6-hydroxydopamine or a similarly related compound may contribute negatively to the external environment that surrounds the DA neuron since DA is susceptible to hydroxyl radical (secondary oxidant) attack (Cohen, 1984). A more interesting scenario, however, lies with 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is the intermediate DA metabolite that has been shown to be neurotoxic (Burke et al, 2003). To demonstrate that it is DOPAL and not DA that is neurotoxic, Burke and

colleagues (Burke et al, 2003) injected varying concentrations of both compounds into the SNpc of rats. These researchers showed that DOPAL was 5-10 times more neurotoxic than DA. Thus, in the extracellular space, because MAO-B metabolizes DA to DOPAL (Fornai et al, 2000; Burke et al, 2004), DA via DOPAL, possibly contributes to changes in the extracellular milieu. DOPAL may also be the reason why DA terminals are severely damaged.

MPTP, Superoxide and the DA Intraneuronal Environment.

DA neurons, as abundant as they may be in the SNpc, are a very fragile lot and are likely a victim of their own environment. The major organelle within the DA neuron that produces the lion's share of superoxide radicals is the mitochondrion (Beal, 2003). This organelle controls oxidation-reduction reactions and is the major source of cellular energy through its respiratory chain and oxidative phosphorylation reactions (Przedborski and Jackson-Lewis, 2000). At the complex I site of the mitochondrial respiratory chain (METC), the superoxide radical is released into the cytosol where, under physiological conditions, it is controlled by the manganese form of SOD (MnSOD), which is located in the internal membrane of the mitochondrion (Keller et al, 1998). Many investigators have found a decrease in complex I in various tissues including brain tissue from PD patients (Mizuno et al, 1989; Shapira, 1990). Thus, low activity of complex I in the METC translates to increased production of superoxide radicals, a depletion of MnSOD and an oxidative stress within the DA neuron. The relevance of this

scenario to PD is not well understood because it is not clear whether the deficit in complex I is or is not a cause of PD.

Once MPP+ exits the glial cells, it is taken up from the extracellular space into the DA neuron via the DA transporter (DAT) (Javitch et al, 1985; Bezard et al, 1999). Although recent evidence shows that these transporters are injured during the uptake process (Jakowec et al, 2004), enough of them remain to transport MPP+ into the cytosol of the DA neuron. DAT are absolutely necessary for the MPTP neurotoxic process as several groups (Gainetdinov et al, 1997; Bezard et al, 1999) have shown that MPTP does not harm mice lacking DAT. In the cytosol of the DA neuron, when MPP+ is not taken up into the vesicles, MPP+ can assist in altering the internal environment of the DA neuron by blocking the METC at the complex I site (Nicklas et al, 1985; Nicklas et al, 1987). This, in turn, kicks out an overabundance of superoxide radicals which apparently can no longer be controlled by MnSOD. Klivenyi and colleagues (Klivenyi et al, 1998) have shown that, as long as sufficient stores of MnSOD are present: 1) mice are protected against the damaging effects of MPTP; and 2) the superoxide radical influences the internal environment of the DA neuron. Furthermore, MPP+ has also been shown to affect complex III (Mizuno et al, 1988) such that the increased production of the superoxide radical here also contributes to the disruption of the normal cytosolic environment within the SNpc DA neuron.

MPTP, NO and the Neuronal Intracellular Environment.

Although the superoxide radical does disturb the internal environment of the DA neuron, it is, by itself, not overwhelmingly toxic. In the internal milieu of the SNpc DA neuron, aside from affecting the METC, MPP+ has been demonstrated to increase the expression of the cyclooxygenase-2 (COX-2) enxyme (Teismann et al, 2003). COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to PGH_2 which is then further metabolized to PGE_2 (O'Bannion, 1999). The NO present in the SNpc DA neuron following MPTP administration most likely enters the DA neuron after having traveled some distance from its non-DA neurons in the SNpc that contain nNOS. When both the superoxide radical and NO are in excess in the internal milieu of the DA neuron after MPTP exposure, PGE_2 catalyzes the reaction between these two relatively mildly toxic compounds to produce the secondary oxidant peroxynitrite (Ischiropoulos and al-Mehdi, 1995; Przedborski and Vila, 2003) which again creates a severely hostile environment for the DA neuron. Peroxynitrite nitrates internal cellular components such as enzymes, fatty acids, proteins, lipids, amino acids and DNA (Radi et al, 2002) of which one of the most important of these is the tyrosine hydroxylase (TH) enzyme. This enzyme is the rate-limiting enzyme in the synthesis of DA and is either down-regulated or damaged in PD and in the MPTP model such that the production of DA is severely compromised (Przedborski et al, 1997).

DA and the Internal Neuronal Environment.

In the internal metabolism of the DA neuron, DA can be oxidized to DA-o-quinone and further to 5-cysteinyldopamine (Hastings, 1995). Aside from having a role in peroxynitrite formation through its stimulation of PGE_2, the COX-2 enzyme can facilitate the oxidation of DA which can damage protein-bound sulfhydryl groups (Hastings, 1995). Using HPLC analysis, Teismann et al (2003) showed that MPTP administration elevates ventral midbrain 5-cysteinyldopamine, which is considered a stable modification of DA and evidence that the formation of DA-o-quinone has occurred. DA-o-quinone can contribute to the upheaval of the internal neuronal environment through glutathione depletion and the inactivation of TH (Kuhn et al,1999). On the other hand, while DA is metabolized to DOPAL extraneuronally by MAO-B, within the neuron, DOPAL is formed by MAO-A (Burke et al, 2004). Furthermore, DOPAL is the major metabolite of DA in the human brain (Burke et al, 1999) and levodopa, the drug of choice in the treatment of PD, has been shown to elevate significantly levels of DOPAL in the brain (Fornai et al, 2000). As stated earlier, DOPAL has been shown to destroy the DA neuron at concentrations much lower than DA itself (Burke et al, 2003). Whether MPTP can elevate DOPAL levels in the brain remains to be determined.

Conclusions

Environment plays a significant role in the well-being of the DA neuron. Several cell types including glia and the compounds that these cells secrete work together to maintain an environment suitable for DA neuron survival. Yet, at the same

time, these same cells and agents, when perturbed, can contribute to the death of the DA neuron through reactions which alter their physiological concentrations in both the external and internal environments thus putting the DA neuron in a compromised (oxidative stress) situation. Interestingly, the major players in both environments are relatively the same as is their interplay. Thus, DA, superoxide and NO may all conspire to keep the DA neuron in a highly sensitive state, and when presented with a catalyst, i.e. MPTP, this sensitivity can shift to vulnerability.

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D-β-Hydroxybutyrate rescues mitochondrial respiration and mitigates features of Parkinson disease

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Parkinson disease (PD) is a neurodegenerative disorder characterized by a loss of the nigrostriatal dopaminergic neurons accompanied by a deficit in mitochondrial respiration. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes dopaminergic neurodegeneration and a mitochondrial deficit reminiscent of PD. Here we show that the infusion of the ketone body D-β-hydroxybutyrate (DβHB) in mice confers partial protection against dopaminergic neurodegeneration and motor deficits induced by MPTP. These effects appear to be mediated by a complex II–dependent mechanism that leads to improved mitochondrial respiration and ATP production. Because of the safety record of ketone bodies in the treatment of epilepsy and their ability to penetrate the blood-brain barrier, DβHB may be a novel neuroprotective therapy for PD.

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Introduction

Parkinson disease (PD) is the second most common neurodegenerative disease after Alzheimer disease (1). PD is clinically characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (2), and its main neuropathological feature is the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons (3).

While PD is a sporadic condition of uncertain etiology (2), several lines of evidence suggest that a defect in oxidative phosphorylation contributes to its pathogenesis. For instance, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a neurotoxin that blocks complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial electron transport chain (4), recapitulates in humans the hallmarks of PD (5). Furthermore, reduction in complex I activity has been reported in PD tissues (reviewed in ref. 6). This defect is not confined only to the brain (7), since low complex I activity has also been found in platelets from PD patients (8, 9) and in *cybrid* cells engineered to contain mitochondria derived from platelets of patients suffering from PD (10).

D-β-Hydroxybutyrate (DβHB) is a ketone body produced by hepatocytes and, to a lesser extent, by astrocytes (11). It is an alternative source of energy in the brain when glucose supply is depleted such as during starvation (12). In vitro DβHB prevents neuronal damage seen following glucose deprivation (13) and mitochondrial poison exposure (14). Herein, we show that DβHB infusion protects SNpc dopaminergic neurons against MPTP in a dose-dependent and stereospecific manner and prevents the development of PD-like motor abnormalities in mice. We also provide in vivo and in vitro evidence that DβHB protects not by alleviating MPTP-related complex I inhibition, but by enhancing oxidative phosphorylation via a mechanism dependent on mitochondrial complex II (succinate-ubiquinone oxidoreductase).

Methods

Animals and treatment. All animals were 8- to 10-week-old male C57BL mice (Charles River Laboratories, Wilmington, Massachusetts, USA). Mice were divided into four groups: vehicle (i.e., saline), DβHB, L-hydroxybutyrate (LβHB), and DβHB plus 3-nitropropionic acid (3-NP). Vehicle, DβHB (1.6, 0.8, or 0.4 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich, St. Louis, Missouri, USA), and LβHB (1.6 mmol/kg/d in saline, pH 7.4; Sigma-Aldrich) were administered subcutaneously (1 μl/h) using Alzet mini-osmotic pumps (DURECT Corp., Cupertino, California, USA). 3-NP (Sigma-Aldrich;

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Nonstandard abbreviations used: Parkinson disease (PD); substantia nigra pars compacta (SNpc); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP); D-β-hydroxybutyrate (DβHB); L-B-hydroxybutyrate (LβHB); 3-nitropropionic acid (3-NP); tyrosine hydroxylase (TH); 1-methyl-4-phenylpyridinium (MPP^+); carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); transmembrane potential ($\Delta\psi_m$); arbitrary fluorescence unit (AFU); dihydroxyphenylacetic acid (DOPAC); homovanillic acid (HVA); reactive oxygen species (ROS); tricarboxylic acid (TCA).

15 mg/kg, in 0.1 M PBS adjusted to pH 7.4) was given intraperitoneally 2 hours before the implantation on the first day and then once a day until the animals were sacrificed. This dosage of 3-NP was selected to inhibit complex II but not to induce cell loss. After surgery, animals were allowed to rest for 1 day. Each mouse was then randomly assigned to receive four intraperitoneal injections of either MPTP (18 mg/kg of free base in saline; Sigma-Aldrich) or saline at 2-hour intervals.

Tyrosine hydroxylase immunostaining and quantitative morphology. Seven days after the last MPTP injection, mice were killed and their brains were processed for immunohistochemical studies. Sections (30 μm) were incubated with a polyclonal anti-tyrosine hydroxylase (TH; 1,000 dilution; Calbiochem-Novabiochem Corp., San Diego, California, USA) for 48 hours at 4°C. Biotinylated secondary antibodies followed by avidin-biotin complex were used. Immunoreactivity was visualized by incubation in 3,3′-diaminobenzidine/glucose/glucose oxidase. Total numbers of TH-positive neurons in SNpc were counted stereologically using the optical fractionator method (15). Striatal OD of TH immunostaining, determined by the Scion Image program (Scion Corp., Frederick, Maryland, USA), was used as an index of striatal density of TH innervation (16). The concentration of anti-TH antibody and 3,3′-diaminobenzidine (DAB) and the duration of incubation of striatal sections in DAB were optimized to fall within the linear range of the plot of the immunostaining intensities and the scanned ODs.

Measurement of DβHB and succinate levels. At different time points after the implantation of the osmotic pumps, blood was collected from tails, and brains were quickly removed, freeze-clamped under liquid nitrogen, and stored at -80°C. Frozen tissues were treated with perchloric acid and neutralized with sodium hydroxide as previously described (17). Both DβHB and succinate were measured spectrophotometrically at 340 nm using commercial kits from Sigma-Aldrich and from Roche Molecular Biochemicals (Indianapolis, Indiana, USA) respectively, following the manufacturers' instructions.

Measurement of striatal 1-methyl-4-phenylpyridinium levels. Mice infused with either saline or DβHB (1.6 mmol/kg/d) were injected with MPTP (18 mg/kg) as described above and killed 90 minutes after the fourth injection. HPLC with UV detection (295 nm) was used to measure striatal 1-methyl-4-phenylpyridinium (MPP+) levels as previously described (18) with the following modifications: a reverse-phase Altima C18 column (Alltech Associates Inc., Deerfield, Illinois, USA) and a mobile phase consisting of 89% 50 mM KH_2PO_4 and 11% acetonitrile were used. Data represent mean ± SEM of five mice per group.

Synaptosomal uptake of MPP^+. Striata were dissected out from naive mice and processed for uptake experiments as described previously (19) with a few modifications. Briefly, striata were homogenized in 0.32 M sucrose and centrifuged at 700 *g*, 4°C, for 10 minutes. The supernatant was removed and centrifuged at 27,000 *g* for 30 minutes. The resulting synaptosomal pellet was suspended at 1.2 mg/ml (original wet weight) in Krebs-Ringer phosphate buffer (pH 7.4). The uptake reaction was initiated by addition of 0.6 mg of synaptosomes to tubes containing [^{3}H]MPP^+ (~4 nM, ~800,000 degradations per minute, specific activity 31.6 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Missouri, USA) in the absence or presence of DβHB (up to 5 mM) at 37°C for 6 minutes. Nonspecific uptake was assessed in the presence of 10 μM mazindol. Data represent mean ± SEM of three mice per group.

Isolation of brain mitochondria. Brains from C57BL mice were homogenized in isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM HEPES, and 2 mg/ml fat-free BSA) using a motorized Dounce homogenizer with eight up-and-down strokes. The homogenate was centrifuged at 1,000 *g* for 10 minutes, and the resulting supernatant was layered onto 5 ml of 7.5% Ficoll medium on top of 5 ml of 10% Ficoll medium and centrifuged at 79,000 *g* for 30 minutes (the Ficoll medium contained 0.3 M sucrose, 50 μM EGTA, and 10 mM HEPES). The mitochondrial pellet was resuspended in isolation buffer. Protein concentrations were determined by the bicinchoninic assay (Pierce Chemical Co., Rockford, Illinois, USA) method with BSA as a standard protein.

Mitochondrial accumulation of MPP^+. Brain mitochondria were isolated and resuspended in buffer as described previously (20) but with a few modifications. The uptake reaction was initiated by addition of 0.6 mg of mitochondria to tubes containing 5 μM [^{3}H]MPP^+ and 45 μM MPP^+ in the absence or presence of DβHB (up to 5 mM) at 25°C for 3 minutes. Nonspecific uptake was assessed in the presence of 5 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Data represent mean ± SEM of four or five mice per group.

Polarography. Brain mitochondria were suspended in respiration buffer consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM HEPES, 5 mM K_2HPO_4, and freshly added 1 mg/ml defatted BSA at 30°C, and oxygen-consumption rates were measured in a closed-chamber cuvette with a mini-stirring bar using a Clark-type electrode (Hansatech Instruments Ltd., Norfolk, United Kingdom). For each reading, 300 μg protein was used in a final 1-ml respiration buffer, and all mitochondria preparations had an average respiratory control ratio of at least 5 when 10 mM glutamate and 5 mM malate were used as NADH-linked substrates.

ATP measurements. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Where 3-NP was used, it was added from the beginning with MPP^+ (5 minutes) or rotenone (2.5 minutes) to mitochondria before the addition of DβHB. When the reaction was stopped, mitochondrial suspension from the cuvette was lysed in an equal volume of lysis buffer from the ATP biolumi-

nescence assay kit (Roche Molecular Biochemicals), and the content of ATP was measured according to the manufacturer's instructions. Light emitted from luciferase-mediated reaction was captured in a tube luminometer and calculated from a log-log plot of the standard curve of known ATP concentrations.

Measurements of mitochondrial H_2O_2 production. Samples were prepared under conditions identical to those of polarographical study. Mitochondria suspended in respiration buffer were incubated in the presence or absence of different substrates or inhibitors using the same incubation times as those of polarographical study. Phenazine methosulfate (0.1 mM) was used to oxidize NADH (21). Hydrogen peroxide, converted from superoxide by manganese-superoxide dismutase, was measured using 5 μM Amplex red (Molecular Probes, Eugene, Oregon, USA) and 5 U/ml HRP. Fluorescence was detected by a Perkin-Elmer (Boston, Massachusetts, USA) LS55 spectrofluorometer with an excitation wavelength of 550 nm (slit 1.5 nm) and an emission wavelength of 585 nm (slit 3 nm). H_2O_2 production was calculated from a standard curve generated from known concentrations of H_2O_2.

Measurements of mitochondrial transmembrane potential. Safranine, a cationic fluorescence dye accumulated and quenched inside energized mitochondria (22, 23) was used to measure transmembrane potential ($\Delta\psi_m$). Mitochondria were incubated with 10 mM glutamate, 5 mM malate, and 5 μM safranine (Sigma-Aldrich) in respiration buffer 5 minutes before 5 mM DβHB was added, and $\Delta\psi_m$ was monitored for an additional 5 minutes. FCCP (5 μM) was used as a positive control to collapse $\Delta\psi_m$. Fluorescence was detected by a Perkin-Elmer LS55 spectrofluorometer with an excitation wavelength of 495 nm (slit 3 nm) and an emission wavelength of 586 nm (slit 5 nm). Data are reported in arbitrary fluorescence units (AFUs).

Complex I activity. Largely based on protocols described by Birch-Machin and Turnbull (24), brain mitochondria were lysed by freeze-thawing in hypotonic buffer (25 mM KH_2PO_4 [pH 7.2], 5 mM $MgCl_2$) three times. To initiate the reaction, 50 μg mitochondria were added to the assay buffer (hypotonic buffer containing 65 μM ubiquinone$_1$, 130 μM NADH, 2 μg/ml antimycin A, and 2.5 mg/ml defatted BSA) in the absence or presence of different concentrations of rotenone (2.5–15 μM) or MPP^+ (10–30 mM). The oxidation of NADH by complex I was monitored at 340 nm spectrophotometrically for 3 minutes at 30°C prior to the addition of rotenone (2 μg/ml), after which the activity was measured for an additional 3 minutes. The difference in rate before and after the addition of rotenone (2 μg/ml) was used to calculate complex I activity.

Complex II histochemistry. Animals were injected intraperitoneally with either saline or 3-NP (15 mg/kg) once daily for 8 days, the same regimen used in the animals that received DβHB. As described previously (25), animals were perfused with PBS containing 10% glycerol. Brains were rapidly removed, frozen in dry ice–cooled isopentane, and stored at -80°C. Brains were sectioned at 20 μm throughout the entire nigra and striatum. Sections were mounted onto glass microscope slides, and complex II activity was revealed by incubation of sections at 37°C for 20 minutes in 50 mM phosphate buffer (pH 7.6) containing 50 mM succinate as a substrate and 0.3 mM Nitroblue tetrazolium (NBT) as an electron acceptor.

Immunoblots. Total tissue proteins from ventral midbrains of MPTP- and saline-treated mice were isolated as described previously (26), and 20 μg proteins were separated on 12% SDS-PAGE. Membranes were blotted with polyclonal anti–β-hydroxybutyrate dehydrogenase (1:100; a generous gift from Andrew Marks, Columbia University, New York, New York, USA) and monoclonal anti–β-actin (1:5,000) overnight at 4°C. Secondary antibodies conjugated with HRP were used. Bands of interest were analyzed and quantified using FluorChem 8800 (Alpha Innotech Corp., San Leandro, California, USA).

Rotarod performance. The Economex system (Columbus Instruments, Columbus, Ohio, USA), consisting of four rotating rods of 3 cm diameter in separated compartments, enables four mice to be recorded simultaneously. Seven days after MPTP or saline injections, implanted pumps containing 1.6 mmol/kg/d DβHB were removed, and mice (4–13 animals per group) were allowed to recover from surgery and dehydration for an additional 7 days. On the testing day, animals were first pretrained three times (1 hour apart) using an accelerating mode. After these training sessions, the time on the rod, with a maximum recording time of 240 seconds, was recorded for successive rotational speeds (15, 18, 21, 24, 27, 30, 32, 36, and 40 rpm), and the overall rod performance (ORP) for each mouse was calculated by the trapezoidal method as the area under the curve in the plot of time on the rod versus rotation speed (27). To assess the responsiveness of the MPTP-related motor deficit to dopaminergic stimulation, mice were injected intraperitoneally with L-3,4-dihydroxyphenylalanine (L-DOPA) methyl ester/benserazide (100/25 mg/kg), and Rotarod performance was assessed 45 minutes later.

Measurement of dopamine and its metabolite levels in striatal and ventral midbrain tissues. Animals from the Rotarod study were sacrificed, and their striata and ventral midbrains were dissected out and stored at -80°C until analysis. On the day of the assay, striatal and ventral midbrain tissues were sonicated in 50 and 10 volumes (wt/vol), respectively, of 0.1 M perchloric acid containing 50 ng/ml dihydrobenzylamine as internal standard. After centrifugation at 15,000 *g* for 15 minutes at 4°C, 20 μl of supernatant was injected onto a C18 reverse-phase HR-80 catecholamine column (ESA Inc., Bedford, Massachusetts, USA). The mobile phase consisted of 94% 50 mM sodium phosphate/0.2 mM EDTA/1.2 mM heptanesulfonic acid (pH 3.2) solution and 6% methanol. The flow rate was 1.5 ml/min. Peaks were detected by an ESA 8 Channel CoulArray system. Data were collected and processed using the CoulArray data analysis program (version 1.12).

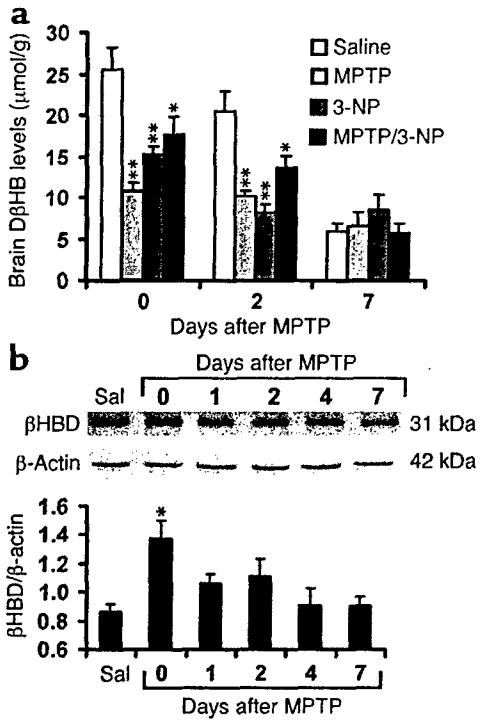

Figure 1
Brain levels of DβHB and β-hydroxybutyrate dehydrogenase (βHBD) under different treatments. (**a**) One day after implantation of pumps containing DβHB, animals were injected intraperitoneally with saline (Sal), MPTP, or 3-NP as described in Methods, and brain levels of DβHB were measured at 0 days (90 minutes after the fourth injection), 2 days, and 7 days thereafter. The utilization of DβHB was increased when cells were under metabolic stress induced by these toxins. $n = 4$–6; $^*P < 0.05$ and $^{**}P < 0.01$ compared with the respective control saline groups. (**b**) Western blot analysis of ventral midbrains from MPTP-intoxicated mice shows upregulation of this enzyme as early as day 0. $n = 4$–5 per group; $^*P < 0.05$ compared with the control saline group. β-Actin is used to normalize βHBD values.

Statistical analysis. All values are expressed as mean ± SEM. Differences between means were analyzed using one-way ANOVA followed by Newman-Keuls post-hoc testing for pairwise comparison. The null hypothesis was rejected when P was greater than 0.05.

Results

MPTP upregulates DβHB-metabolizing enzyme and increases utilization of DβHB in the brain. To assure sustained high tissue levels of DβHB during the experiment, this short-half-life (28) compound was infused subcutaneously at a dose of 1.6 mmol/kg/d for the entire 7 days. This regimen seemed well tolerated and yielded a stable plasma level of approximately 0.9 mM throughout the 7-day period. Likewise, brain DβHB levels in mice intoxicated with MPTP did not significantly change throughout the experiment (Figure 1a). Brain DβHB levels in mice that received saline instead of receiving MPTP, 3-NP, or both were significantly higher, at least at the beginning of the experiment (Figure 1a).

Circulating DβHB readily crosses the blood-brain barrier and enters mitochondria, where it is metabolized by β-hydroxybutyrate dehydrogenase to acetoacetate; the latter is converted to acetyl-CoA, which feeds into the Krebs cycle (29). In saline-injected control mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain (the brain region that contains the SNpc) was detectable (Figure 1b). In MPTP-injected mice, β-hydroxybutyrate dehydrogenase protein content in ventral midbrain rose rapidly and remained elevated for 2 days after the last injection of MPTP (Figure 1b). These data suggest that MPTP-related cellular stress is associated with a β-hydroxybutyrate dehydrogenase upregulation, which in turn may facilitate utilization of DβHB in the brain.

DβHB attenuates MPTP-induced dopaminergic neurodegeneration. One day after implantation of pumps containing either vehicle or DβHB, mice were injected with MPTP. Seven days later, the brains of these animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. In saline-injected mice infused with either vehicle or DβHB, numbers of TH-positive neurons in the SNpc were identical (Table 1; Figure 2, a and b), as were TH ODs in the striatum (Table 1; Figure 2, i and j). In MPTP-injected mice infused with vehicle, there was an approximately 70% loss of SNpc TH-positive neurons and an approximately 90% reduction of striatal TH ODs (Table 1; Figure 2, e and m) compared with saline-injected controls (Table 1; Figure 2, a and i). In contrast, in MPTP-injected mice

Table 1
TH- and Nissl-positive neurons in SNpc and striatal TH density

	MPTP	3-NP	Nigral TH	Nigral Nissl	Striatal TH OD (×100)
Vehicle	–	–	9,770 ± 694	15,525 ± 930	21.78 ± 1.90
DβHB (1.6 mmol/kg/d)	–	–	9,293 ± 590	14,880 ± 416	23.76 ± 2.10
LβHB (1.6 mmol/kg/d)	–	–	9,040 ± 705	12,987 ± 1,274	20.47 ± 1.43
Vehicle	–	+	8,933 ± 1,040	12,387 ± 1,169	23.11 ± 4.43
Vehicle	+	–	3,233 ± 280	6,445 ± 380	1.61 ± 0.16
Vehicle	+	+	2,600 ± 654	5,860 ± 850	1.76 ± 0.10
DβHB (0.4 mmol/kg/d)	+	–	3,168 ± 625	5,392 ± 847	1.80 ± 0.12
DβHB (0.8 mmol/kg/d)	+	–	3,720 ± 185	7,693 ± 659	2.00 ± 0.39
DβHB (1.6 mmol/kg/d)	+	–	6,300 ± 506[A]	9,597 ± 601	3.73 ± 0.10[B]
LβHB (1.6 mmol/kg/d)	+	–	2,780 ± 236	7,525 ± 360	1.10 ± 0.33
DβHB (1.6 mmol/kg/d)	+	+	1,947 ± 389	4,627 ± 701	1.73 ± 0.27

Animals with pumps containing either vehicle (saline) or different isoforms of β-hydroxybutyrate were injected intraperitoneally with MPTP, 3-NP, or saline (not shown). Data represent mean ± SEM of six to nine mice per group. [A]$P < 0.01$ and [B]$P < 0.05$ compared with the saline-MPTP group.

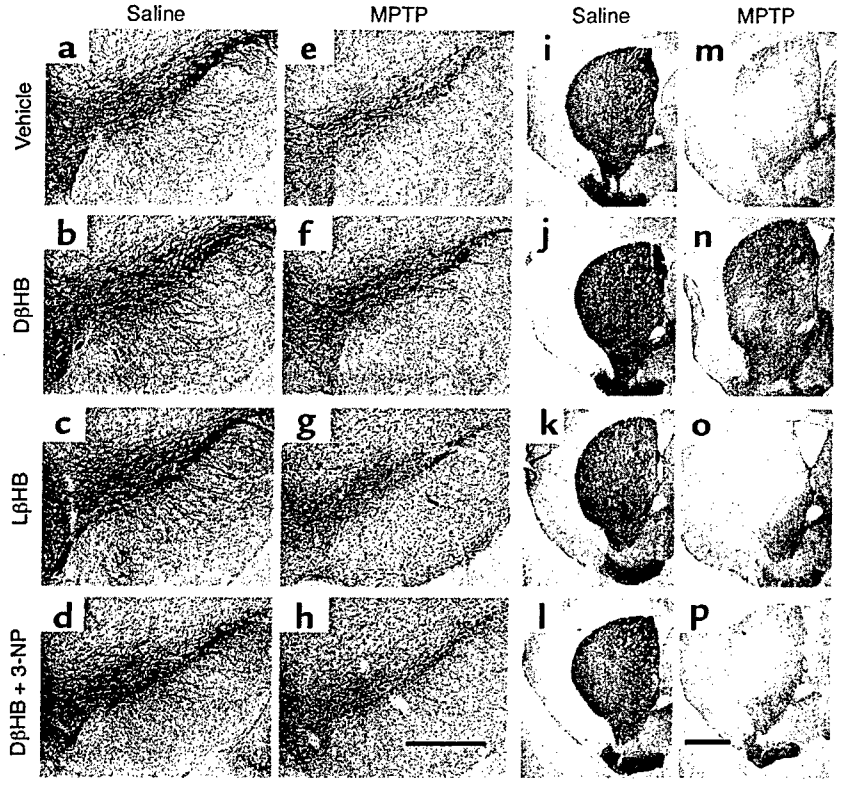

Figure 2
Protective effect of DβHB against MPTP-induced neurodegeneration. (**a–h**) TH-positive neurons in SNpc, and (**i–p**) TH-positive terminals in striatum. Animals were infused subcutaneously with vehicle (saline; **a**, **e**, **i**, and **m**), DβHB (1.6 mmol/kg/d; **b**, **d**, **f**, **h**, **j**, **l**, **n**, and **p**), or LβHB (1.6 mmol/kg/d; **c**, **g**, **k**, and **o**) 1 day before receiving intraperitoneal injections of either saline (**a–d** and **i–l**) or MPTP (18 mg/kg; **e–h** and **m–p**). There is an extensive loss of TH-positive neurons (**e**) and terminals (**m**) in MPTP-injected animals. This loss is attenuated by DβHB (**f** and **n**) but not by its inactive isomer LβHB (**g** and **o**). The complex II inhibitor 3-NP was given intraperitoneally (15 mg/kg) daily for the entire period of DβHB infusion. In the presence of 3-NP, DβHB does not confer neuroprotection. Scale bars: 500 μm (**a–h**) and 1 mm (**i–p**). Please refer to Table 1 for quantification of neurons and terminals in each animal group.

infused with DβHB, less reduction in SNpc TH-positive neurons and striatal TH ODs was observed (Table 1; Figure 2, f and n). To control for the specificity of DβHB neuroprotection, another set of MPTP-injected mice received infusion of the inactive isomer LβHB. In these mice, the loss of dopaminergic neurons was as severe as in mice infused with vehicle (Table 1; Figure 2, g and o). Thus, DβHB, but not its inactive isomer, can attenuate neurotoxic effects of MPTP on dopaminergic cell bodies in the SNpc and nerve fibers in the striatum.

DβHB attenuates the loss of dopamine and the motor deficit induced by MPTP. To examine whether DβHB protects not only against structural damage but also against functional deficits caused by MPTP, we assessed levels of dopamine and two of its metabolites, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in ventral midbrain and striatum, as well as locomotor activity, in these animals. In MPTP-injected mice that did not receive DβHB, there was a reduction in dopamine and its metabolites (Table 2) in both ventral midbrain and striatum. Behaviorally, the length of time that these MPTP-injected mice remained on the rotating rods was significantly shorter than that of the saline-injected controls (Figure 3). The motor deficit observed in MPTP-treated mice was alleviated by the administration of L-DOPA/benserazide (data not shown), indicating that this motor deficit results from a loss of dopamine. In MPTP-injected mice that did receive DβHB, the levels of dopamine and its metabolites were all significantly higher than those in MPTP-injected mice that did not receive DβHB (Table 2). Of note, the attenuation of MPTP-induced dopamine loss by DβHB was smaller than the attenuation of MPTP-induced SNpc neuronal death by DβHB. Similarly, MPTP-injected mice that received DβHB performed much better on the rotating rods than MPTP-injected mice that did not receive DβHB (Figure 3). Saline-injected mice that received DβHB had similar levels of dopamine and metabolites (Table 2) and simi-

Table 2
Levels of dopamine and its metabolites in ventral midbrain and striatal tissues

	Ventral midbrain levels (ng/mg tissue)			Striatal levels (ng/mg tissue)		
	DA	DOPAC	HVA	DA	DOPAC	HVA
Vehicle	0.32 ± 0.01	0.098 ± 0.003	1.07 ± 0.02	15.81 ± 0.69	0.91 ± 0.06	1.41 ± 0.03
DβHB	0.33 ± 0.02	0.104 ± 0.010	1.09 ± 0.11	16.92 ± 0.53	1.02 ± 0.01	1.40 ± 0.11
Vehicle/MPTP	0.17 ± 0.01	0.046 ± 0.003	0.50 ± 0.03	0.86 ± 0.21	0.10 ± 0.02	0.36 ± 0.05
DβHB/MPTP	0.23 ± 0.01[A]	0.070 ± 0.005[A]	0.71 ± 0.05[A]	2.41 ± 0.45[B]	0.24 ± 0.03[B]	0.64 ± 0.04[A]

Animals from the Rotarod study were killed, and their brains were removed and measured by HPLC for the levels of dopamine and its metabolites. Data represent mean ± SEM of 4–13 mice per group. [A]$P < 0.01$; [B]$P < 0.05$ compared with the MPTP-treated group without DβHB.

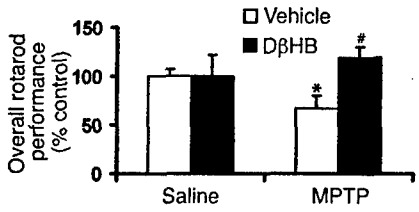

Figure 3

Protective effect of DβHB against motor deficit in MPTP-treated mice. Animals were infused subcutaneously with either vehicle (saline) or DβHB (1.6 mmol/kg/d) 1 day before receiving intraperitoneal injections of either saline or MPTP (18 mg/kg). Pumps were removed at day 7, and animals were allowed to recover from surgery and dehydration for an additional 7 days before their Rotarod performance was assessed. Motor deficit is observed in the MPTP-treated animals, but DβHB significantly improves this impairment. DβHB does not affect base-line motor function in saline-injected mice. $n = 4$–13; $^*P < 0.05$ compared with the saline-vehicle group; $^\#P < 0.05$ compared with the MPTP-vehicle group.

lar motor performance (Figure 3) to those of saline-injected mice that did not receive DβHB.

DβHB does not affect MPTP activation. MPTP is a protoxin whose effect correlates with the striatal content of its active metabolite MPP^+ (30). Striatal levels of MPP^+ 90 minutes after the last injection of MPTP did not differ between mice that received DβHB (30.9 ± 1.6 μg/g tissue) or vehicle [26.8 ± 1.3 μg/g tissue; Student's t test with 6 degrees of freedom ($t(6)$) = 1.98; $P = 0.1$]. MPTP-induced dopaminergic neurotoxicity relies on the entry of MPP^+ into dopaminergic neurons via dopamine transporters (31). DβHB did not impair the uptake of [^{3}H]MPP^+ by striatal synaptosomes at concentrations up to 5 mM, which is more than five times the plasma concentration found in DβHB-infused animals (vehicle, 100% ± 2.3% of control; DβHB, 99.1% ± 1.8% of control; $t(6) = 0.3$; $P = 0.8$). Inside dopaminergic neurons, MPP^+ is concentrated within mitochondria by a mechanism that depends on mitochondrial $\Delta\psi_m$ (20). At 5 mM, DβHB did not alter the uptake of [^{3}H]MPP^+ by purified brain mitochondria (vehicle, 100% ± 4.1% of control; DβHB, 93.2% ± 0.5% of control; $t(6) = 1.7$; $P = 0.1$). Thus, it is unlikely that the neuroprotective effect of DβHB in the MPTP model of PD results from alterations in the key MPTP toxicokinetic steps described above.

DβHB increases mitochondrial oxygen consumption. DβHB has been used as a mitochondrial substrate (32, 33). We thus asked whether DβHB could support oxidative phosphorylation in brain mitochondria, and, if so, whether it may rescue mitochondrial respiration depressed by MPP^+-mediated complex I blockade (34). Consistent with DβHB being a mitochondrial substrate, we found that it increased oxygen consumption in a dose-dependent manner (Figure 4, a and b). The effects of DβHB in supporting mitochondrial respiration are stereospecific, since the inactive isomer LβHB failed to improve oxidative phosphorylation (Figure 4c). We also found that DβHB ameliorated oxygen consumption impaired by different concentrations of MPP^+ (Figure 4a) and of another complex I inhibitor, rotenone (Figure 4b). At 25 μM MPP^+ and 25 nM rotenone, which we found to inhibit about 25% of the oxygen consumption in glutamate- and malate-supported mitochondria, DβHB restored completely the oxygen consumption depressed by these inhibitors (Figure 4, a and b). At 100 μM MPP^+ and 100 nM rotenone inhibits more than 90% of the oxygen consumption in glutamate- and malate-supported mitochondrial respiration (data not shown). At these concentrations, DβHB restored completely the oxygen consumption inhibited by MPP^+, but only partially that inhibited by rotenone (Figure 4, a and b).

DβHB does not uncouple mitochondria. To assure that the increase in rate of oxygen consumption induced by DβHB is not an artifact of uncoupled mitochondria, we measured $\Delta\psi_m$. As expected, the uncoupler FCCP at 5 μM collapsed the $\Delta\psi_m$ in isolated mitochondria (FCCP, 419 ± 23 AFUs; no FCCP, 69 ± 2 AFUs). Conversely,

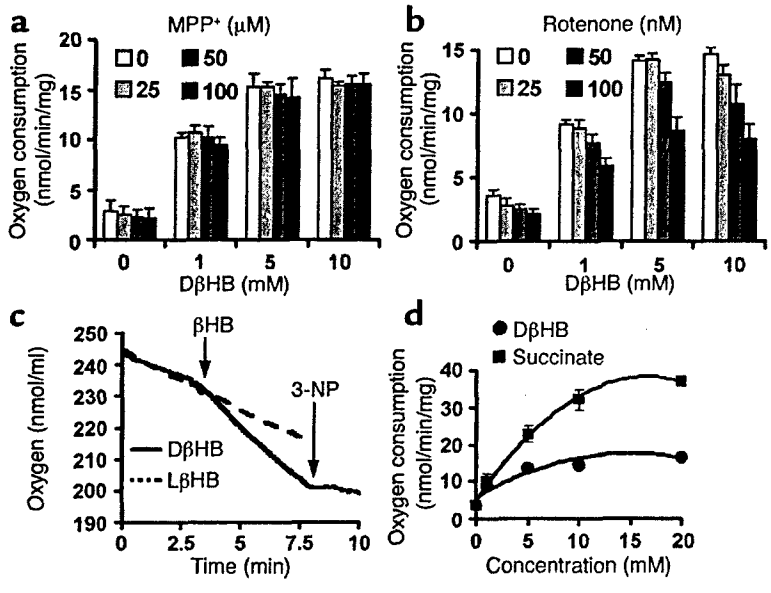

Figure 4

DβHB increases oxygen consumption in purified brain mitochondria. Mitochondria (300 μg) were incubated in the absence or presence of MPP^+ (5 minutes; **a**) or rotenone (2.5 minutes; **b**) at 30°C, and then 5 mM DβHB was added to induce oxygen consumption. DβHB attenuated inhibition of mitochondrial respiration induced by MPP^+ (**a**) or rotenone (**b**) at indicated concentrations, which blocked about 25–90% of oxygen consumption when glutamate and malate were used as NADH-linked substrates (data not shown). (**c**) The improvement of oxygen consumption by DβHB is stereospecific and is blocked by 10 mM 3-NP, a complex II inhibitor. (**d**) DβHB increases oxygen consumption in a dose-dependent and saturable fashion as seen with succinate, a complex II substrate, although not as efficiently as succinate does on an equimolar basis. $n = 3$–4.

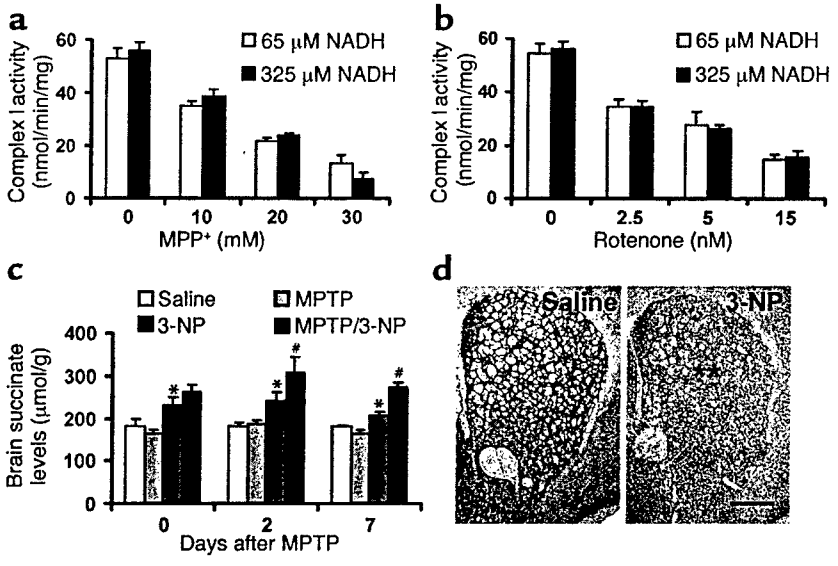

Figure 5

Dose-response study of NADH in complex I activity (**a** and **b**) and brain levels of succinate (**c**). In mitochondria lysed by freeze-thawing, when the inhibition of complex I activity was titrated with different concentrations of MPP^+ (**a**) or rotenone (**b**), different amounts of NADH did not produce different responses in complex I activity (n = 4 per group). (**c**) Levels of succinate were measured in the brains of animals treated with 18 mg/kg/d MPTP or 15 mg/kg 3-NP, or both. Levels of succinate in the group that received DβHB (1.6 mmol/kg/d) are significantly increased in the presence of 3-NP. n = 3–10 per group; $^*P < 0.05$ compared with the control saline group; $^\#P < 0.05$ compared with the 3-NP group. (**d**) Histochemical analysis in striatal sections shows that when animals were treated with 3-NP (right panel) at this concentration for 8 days, there was approximately 40% reduction in complex II activity in the striatum compared with that in the group treated with saline (left panel). n = 5 per group; $^{**}P < 0.01$. Scale bar: 500 μm.

DβHB at concentrations as high as 5 mM had no effect on mitochondrial $\Delta\psi_m$ (DβHB, 68.58 ± 3.07 AFUs; no DβHB, 65.21 ± 3.03 AFUs; n = 5 per group; $P > 0.05$). We also found that the increase in oxygen-consumption rate produced by DβHB could be blocked by antimycin A, a complex III inhibitor (base line, 4.49 ± 0.62 nmol/min/mg; DβHB, 14.14 ± 0.43 nmol/min/mg; DβHB + antimycin A, 5.99 ± 0.95 nmol/min/mg; n = 3 per group; $P > 0.05$ comparing base line with the DβHB + antimycin A group). These experiments indicate that DβHB does not uncouple mitochondria at concentrations that increased oxygen consumption.

Effects of DβHB on mitochondrial respiration seem driven by complex II. One product generated from the metabolism of DβHB is NADH, which provides the driving force for the mitochondrial respiration through complex I. Can an increase in availability of NADH compensate for the loss of oxygen consumption due to complex I inhibition? To test this possibility, freeze-thawed disrupted brain mitochondria were incubated with MPP^+, or rotenone, and NADH. Concentrations of MPP^+ and rotenone were selected to produce complex I inhibition ranging from about 40% to 100%, and supplementation of NADH ranged from 0.5 to 2.5 times the normal concentration used in the assay (Figure 5, a and b). These changes in NADH supplementation did not modify the degree of complex I inhibition (Figure 5, a and b). This indicates that DβHB-derived NADH cannot explain the improvement seen in mitochondrial respiration produced by DβHB.

Based on its metabolic pathway, DβHB can also generate succinate, which is capable of stimulating the rate of oxygen consumption in isolated brain mitochondria through complex II. In keeping with this metabolic pathway, we found that both DβHB and succinate did improve oxygen consumption in a dose-dependent and saturable manner, although DβHB was not as potent as succinate (Figure 4d). This is not unexpected, since DβHB has to go through several metabolic steps to generate succinate. In addition, we found that the beneficial effects of DβHB on mitochondrial respiration in the presence of MPP^+ or rotenone were completely abolished by two different complex II inhibitors, 3-NP at 10 mM (Figure 4c) and malonate at 10 mM (data not shown). Together, these data are consistent with the idea that DβHB increases mitochondrial respiration in the face of complex I inhibition by a complex II–dependent mechanism.

DβHB neuroprotection is abrogated by mitochondrial complex II inhibition in vivo. To determine whether our in vitro data are relevant to DβHB neuroprotection seen in vivo, we first measured succinate levels in the brains of DβHB-infused mice. Upon inhibition of complex II, DβHB infusion indeed increased levels of succinate in the brain (Figure 5c). Next, MPTP-injected mice infused with DβHB were injected with 3-NP. This irreversible complex II inhibitor was administered daily for the entire period of DβHB infusion at a dosage of 15 mg/kg/d. As illustrated in Figure 5d, this regimen of 3-NP inhibited approximately 40% of complex II activi-

Table 3

ATP levels in purified brain mitochondria

ATP levels (nmol/mg mitochondrial protein)	
Base line (no substrate)	5.37 ± 0.30
DβHB (5 mM)	76.16 ± 6.11[A]
DβHB plus MPP^+ (100 μM)	90.49 ± 9.73[A]
DβHB plus rotenone (100 nM)	25.96 ± 5.22[B]
DβHB plus MPP^+ plus 3-NP (10 mM)	0.62 ± 0.21
DβHB plus rotenone plus 3-NP	0.73 ± 0.23
LβHB	3.85 ± 0.24

Mitochondrial samples were prepared as in the polarographical studies, and ATP levels were measured using a luciferase kit. Data represent mean ± SEM of four mice per group. $^AP < 0.01$ and $^BP < 0.05$ compared with the base-line endogenous ATP level.

Table 4
H_2O_2 measurements in purified brain mitochondria

Treatment	Mitochondrial H_2O_2 production (pmol/min/mg protein)
DβHB (5 mM)	73.83 ± 8.04
Rotenone (100 nM)	132.39 ± 19.68
DβHB plus rotenone	506.00 ± 40.47[A]
DβHB plus rotenone plus 3-NP (10 mM)	522.76 ± 62.23[A]
DβHB plus rotenone plus PM (0.1 mM)	160.50 ± 20.62
LβHB (5 mM) plus rotenone	105.91 ± 7.45
MPP^+ (500 μM)	55.24 ± 12.98
DβHB plus MPP^+	94.92 ± 6.79[B]
DβHB plus MPP^+ plus PM	73.76 ± 6.38
LβHB plus MPP^+	54.28 ± 4.93

Mitochondrial samples similar to those in the polarographical studies were prepared, and the fluorescence dye Amplex red was used to measure H_2O_2 converted from superoxide. Data represent mean ± SEM of four mice per group. [A]$P < 0.01$ compared with the rotenone-alone group; [B]$P < 0.05$ compared with the MPP^+-alone group. PM, phenazine methosulfate.

ty in the striatum without causing cell death in either the SNpc (Table 1) or the striatum, as evidenced by TH or Nissl staining (Table 1; data not shown for striatal Nissl staining). As before, DβHB protected against MPTP neurotoxicity in mice that did not receive 3-NP. However, DβHB failed to reduce MPTP-induced dopaminergic neurodegeneration in mice that did receive 3-NP (Table 1; Figure 2, h and p). Supporting the effectiveness of the 3-NP regimen in blocking complex II is our demonstration that succinate levels in the brain were higher in mice that received 3-NP than in those that did not (Figure 5c). Thus, these results are consistent with the hypothesis that complex II is a pivotal mediator in DβHB's neuroprotective effects.

DβHB does not have antioxidant effects but increases ATP production. Inhibition of complex I by MPP^+ and rotenone generates reactive oxygen species (ROS), raising the possibility that the beneficial effects of DβHB are mediated by an antioxidant action, as previously suggested (14). In isolated mitochondria, DβHB did not reduce but stimulated ROS production in the presence of rotenone or MPP^+ (see Table 4). To elucidate the basis of DβHB-related ROS production, 3-NP was added to the incubation mixture (see Table 4). This complex II inhibitor was unable to block the DβHB-related ROS production, thus ruling out the possibility of a reversed flux of electrons from complex II to complex I as the ROS generator (22, 23). Instead, we suspected that the DβHB-related ROS resulted from additional NADH generated by DβHB metabolism. To test this alternative possibility, phenazine methosulfate, a compound that oxidizes NADH (21), was included in the incubation mixture. Consistent with this possibility, phenazine methosulfate abolished ROS production (see Table 4). These data argue against DβHB having antioxidant properties, at least in this in vitro setting.

Inhibition of complex I by MPP^+ and rotenone also impairs ATP production, raising the possibility that the beneficial effects of DβHB are mediated by attenuation of ATP depletion. We thus measured ATP production in isolated brain mitochondria under conditions similar to those of polarographical study. As shown in Table 3, DβHB increased ATP production from a base line of 5.37 ± 0.30 nmol/mg protein to 76.16 ± 6.11 nmol/mg protein. The increase of ATP production was not detected with the inactive isomer LβHB (3.85 ± 0.24 nmol/mg protein). In agreement with the oxygen-consumption data, DβHB prevented the loss of ATP production caused by 100 μM MPP^+ or 100 nM rotenone (Table 3). Yet, upon addition of 3-NP, DβHB-related ATP production was abolished (Table 3). Together, these data are consistent with the contention that the effects of DβHB seen in the polarographical studies correspond to an increase in oxidative phosphorylation.

Discussion

The present study shows that the ketone body DβHB, a crucial alternative source of glucose for brain energy, confers protection against the structural and functional deleterious effects of the parkinsonian toxin MPTP; these include degeneration of SNpc dopaminergic neurons and striatal dopaminergic fibers, loss of striatal dopamine, and PD-like motor deficit. The beneficial effects of DβHB were achieved by its subcutaneous infusion using osmotic mini-osmotic pumps, which, without apparent distress, allowed its reliable continuous delivery to the brain. While DβHB levels in the brain were stable in DβHB-infused mice exposed to MPTP, in mice injected with saline they were higher at the beginning and then dropped during the experimental period of 7 days. Although the basis for these differences remains to be elucidated, it is possible that the utilization of DβHB in the brain increases rapidly following exposure to mitochondrial poisons such as MPTP and augments progressively in normal brain as part of a metabolic adaptation to sustained high DβHB concentrations.

Utilization of DβHB in the brain is contingent on its conversion to acetoacetate by β-hydroxybutyrate dehydrogenase, which is scarce in the adult brain, especially in the basal ganglia (35). The activity of β-hydroxybutyrate dehydrogenase correlates with its protein content (36), and, following MPTP administration, it is upregulated in the ventral midbrain. MPTP-induced β-hydroxybutyrate dehydrogenase upregulation precedes peak dopaminergic neuronal death in this model (37). It can thus be envisioned that β-hydroxybutyrate dehydrogenase activity increases early enough to allow effective utilization of DβHB by the compromised dopaminergic neurons.

A critical step in activation of MPTP is its conversion into MPP^+ by monoamine oxidase (38). The possibility that DβHB infusion confers protection by interfering with monoamine oxidase activity can be ruled out given the fact that brain levels of MPP^+ were similar between mice that received and those that did not receive DβHB. Also arguing against the possibility that DβHB confers protection by impairing MPTP activation is the fact that DβHB attenuates dopaminergic neuronal death in primary ventral midbrain cultures exposed to MPP^+ (14). DβHB also did not interfere with other key aspects of

MPTP metabolism (39), such as entry of MPP^+ into dopaminergic neurons and mitochondria at concentrations as high as 5 mM. Together these data indicate that DβHB protects not by a pre-complex I mechanism but rather by mitigating the deleterious effects of complex I inhibition on the survival of dopaminergic neurons.

In isolated brain mitochondria, DβHB improves oxygen consumption in the presence of the complex I poisons MPP^+ and rotenone. The DβHB effect is dose dependent and stereospecific. The metabolism of DβHB leads to an elevated mitochondrial [NADH]/[NAD^+] ratio due to NADH generated from the conversion of DβHB to acetoacetate and also from the tricarboxylic acid (TCA) cycle, whose turnover is increased by high levels of acetyl-CoA produced by acetoacetate. NADH is used by complex I to drive mitochondrial respiration. DβHB may increase oxygen consumption by fueling mitochondria with NADH. However, in the presence of complex I inhibition by MPP^+ or rotenone, NADH oxidation is impaired and, as shown in this study, an increase in NADH content is unable to alleviate complex I blockade.

In addition to generating NADH, increased TCA turnover, in theory, should also lead to increases in production of other TCA intermediates such as succinate. Here, we show that DβHB infusion does increase brain succinate content. While succinate is a TCA cycle substrate, its oxidation by succinate dehydrogenase is coupled to a transfer of electrons to ubiquinone of the mitochondrial respiratory chain, and thus succinate is routinely used to support oxygen consumption in the presence of complex I blockade. We demonstrate that inhibition of complex II (a) abrogates DβHB-mediated increases in oxygen consumption in isolated mitochondria and (b) abolishes DβHB-mediated protective effects on SNpc dopaminergic neurons and striatal dopaminergic fibers after MPTP administration. Thus, these data strongly support our hypothesis that the beneficial effect of DβHB in the MPTP model of PD involves a complex II–dependent mechanism.

It has been proposed that the ability of DβHB to decrease MPP^+ neurotoxicity in primary ventral midbrain cultures is related to the oxidation of the coenzyme Q couple, which should, by decreasing the semiquinone, decrease ROS production (14). Contrary to this prediction, we found, at least in isolated mitochondria, that rather than decreasing ROS production induced by MPP^+ or rotenone, DβHB enhanced it even further. These findings cast doubt that DβHB protects the nigrostriatal pathway through an antioxidant mechanism. How can DβHB increase ROS? Succinate is the most effective ROS-generating substrate in intact brain mitochondria (22, 23), by stimulating a reversed flux of electrons from complex II to complex I (22, 23). However, rotenone blocks this ROS signal (22, 23); thus, in the context of the present study, in which complex I is inhibited, this mechanism may not be operative. Instead, our data suggest that DβHB-derived NADH, by feeding complex I, increases the accumulation of electrons upstream to the blockade, thereby stimulating ROS production.

Mitochondrial respiration is tightly linked to ATP synthesis (40). It may thus be speculated that DβHB, by restoring oxygen consumption in MPTP-intoxicated animals, may increase ATP cellular stores. Ablation and inhibition of poly(ADP-ribose) polymerase-1 (41, 42) and creatine supplements (43) mitigate MPTP-induced death of dopaminergic neurons in the SNpc by buffering ATP depletion. These studies underscore the importance of ATP deficit in the MPTP neurodegenerative process. In normal rodents, dopaminergic structures represent less than 15% of the cellular elements in the striatum (44) and hardly more in the ventral midbrain. This renders precarious any detection of ATP changes in brain tissues of MPTP-intoxicated mice (45). To avoid this problem, we studied the effects of DβHB on ATP production in isolated brain mitochondria. By this approach, we were able to demonstrate that DβHB does increase ATP levels in both the absence and the presence of complex I inhibitors. Consistent with the oxygen-consumption data, we also found that the stimulation of ATP production by DβHB likely relies on complex II, as inhibitors of this electron transport chain enzyme eliminated the effect. Data generated in isolated mitochondria may only approximate the more complex situation found in vivo. Despite this caveat, we believe that the most parsimonious explanation for DβHB-induced neuroprotection in the MPTP model of PD is that energy crisis is attenuated by an enhancement of oxidative phosphorylation. It is thus tempting to conclude that, under the current DβHB regimen, the benefit due to the improved ATP production overcomes the possible detriment due to the increased ROS formation in this PD model.

The present study demonstrates that modulation of body DβHB levels may be a straightforward neuroprotective strategy for the treatment of neurodegenerative diseases such as PD. Relevant to this view is the demonstration that mice subjected to dietary restriction (e.g., alternate-day fasting) exhibit higher serum DβHB concentrations and are more resistant to kainic acid–induced hippocampus damage (46) and to MPTP-induced SNpc damage (47). At this point, however, the long-term effects of the chronic use of DβHB on the cell metabolism and, especially, on the mitochondrial function are not known. DβHB has been administered orally for several months to two 6-month-old infants with hyperinsulinemic hypoglycemia (48). Despite the high dosage (up to 32 g/d), these patients seem to tolerate quite well. In addition, the ketogenic diets, which result in high levels of DβHB, have been used for more than 70 years in humans as a treatment for refractory epilepsy and have proven safe and well tolerated.

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Inhibition of Calpains Prevents Neuronal and Behavioral Deficits in an MPTP Mouse Model of Parkinson's Disease

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The molecular mechanisms mediating degeneration of midbrain dopamine neurons in Parkinson's disease (PD) are poorly understood. Here, we provide evidence to support a role for the involvement of the calcium-dependent proteases, calpains, in the loss of dopamine neurons in a mouse model of PD. We show that administration of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) evokes an increase in calpain-mediated proteolysis in nigral dopamine neurons *in vivo*. Inhibition of calpain proteolysis using either a calpain inhibitor (MDL-28170) or adenovirus-mediated overexpression of the endogenous calpain inhibitor protein, calpastatin, significantly attenuated MPTP-induced loss of nigral dopamine neurons. Commensurate with this neuroprotection, MPTP-induced locomotor deficits were abolished, and markers of striatal postsynaptic activity were normalized in calpain inhibitor-treated mice. However, behavioral improvements in MPTP-treated, calpain inhibited mice did not correlate with restored levels of striatal dopamine. These results suggest that protection against nigral neuron degeneration in PD may be sufficient to facilitate normalized locomotor activity without necessitating striatal reinnervation. Immunohistochemical analyses of postmortem midbrain tissues from human PD cases also displayed evidence of increased calpain-related proteolytic activity that was not evident in age-matched control subjects. Taken together, our findings provide a potentially novel correlation between calpain proteolytic activity in an MPTP model of PD and the etiology of neuronal loss in PD in humans.

Key words: substantia nigra; dopamine; neurotensin; FosB; protease; adenovirus; behavior; L-Dopa

Introduction

The molecular events responsible for the loss of dopaminergic neuron in the substantia nigra pars compacta (SNc) in Parkinson's disease (PD) remain poorly understood. One prominent feature of PD is a deficiency in mitochondrial function attributed to reduced complex 1 activity in the SNc (Schapira et al., 1989; Greenamyre et al., 2001). Experimentally, administration of chemical inhibitors of complex 1 of the mitochondrial respiration chain can mimic key features of PD, including the selective dopaminergic neuropathology and behavioral deficits (Beal, 2001). These findings support accumulating evidence that nigral dopamine neurons are highly sensitive to stress related to reduced mitochondrial function.

The cellular consequences of deficits in mitochondrial function include reduced ATP production (Greenamyre et al., 1999), oxidation-related changes in protein function (Jenner, 1998; Przedborski et al., 2003), and poor calcium homeostasis (Sheehan et al., 1997; Sherer et al., 2001). It is of particular relevance to this latter point that previous work has also shown that *N*-methyl-4-pyridinium (MPP+), the active metabolite of the dopaminergic neurotoxin *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), can evoke a sustained elevation of cytoplasmic calcium levels (Chen et al., 1995). This change in calcium likely occurs through several processes, including secondary excitotoxic mechanisms and depletion of mitochondrial calcium pools (Frei and Richter, 1986; Kass et al., 1988). Such abnormal calcium homeostasis manifests activation of various intracellular signaling pathways that impact on neuronal function and survival (Wang and Yuen, 1994). Although the effects of increased intracellular calcium in nigral dopamine neurons is almost certainly complex, one consequence may be the activation of the calcium-sensitive proteases, calpains.

Calpains are a highly conserved family of calcium-dependent proteases. There are two ubiquitously expressed calpain isoforms, μ (calpain-1) and m (calpain-2). Each calpain is composed of a unique large subunit and a common small regulatory subunit (for review, see Sorimachi et al., 1997). The importance of calpains is underscored by the observation that mice deficient in the 30 kDa small regulatory subunit suffer embryonic lethality

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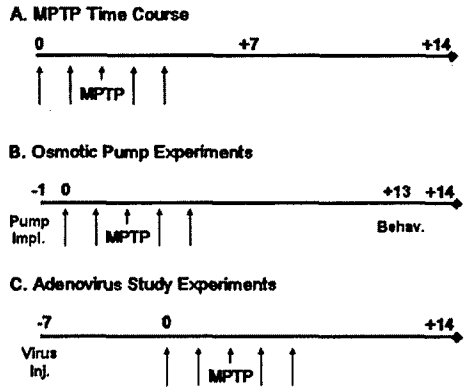

Figure 1. Schematic representation of time course and experimental manipulations for each set of experiments in this study. Each horizontal line represents the duration of each experiment. Numerals above the horizontal line indicate time point (in days) of experimental endpoints, whereas the set of five vertical lines indicates the timing of MPTP dosing (25 mg/kg measured as free base, i.p., per day for 5 consecutive days). *A*, Mice used for experiments examining the time course of calpain activation after MPTP intoxication were taken either before MPTP (day 0) or 7 or 14 d after the first injection (+7, +14). *B*, Mice implanted with osmotic minipumps (Alza) 1 d (−1) before the initiation of the MPTP regime (0). In this set of experiments, the locomotor behavior of these mice was analyzed 1 d before (+13) the 2 week end point (+14), and additional groups were also analyzed for survival at 3 weeks (+21). *C*, Recombinant adenoviruses were administered 1 week (−7) before the start of MPTP dosing (0), and tissues were analyzed 2 weeks later (+14). See Materials and Methods for details.

(Arthur et al., 2000; Zimmerman et al., 2000). In the CNS, calpains are widely expressed (Goto et al., 1994; Li et al., 1996) and modulated by an endogenously expressed inhibitory protein, calpastatin (Emori et al., 1987; Wang and Yuen, 1994).

The role of calpains in PD is unknown. In human PD tissues, it has been reported that expression of m-calpain is increased in dopamine neurons (Mouatt-Prigent et al., 1996); however, the significance of this observation is not clear. Here, we provide evidence to support a role for calpain activation as a process mediating the loss of nigral dopamine neurons in a mouse model of PD and demonstrate that inhibition of calpains prevents reduced motor function in mice through normalization of basal ganglia (BG) activity, albeit in the absence of restored striatal dopamine. We also provide evidence that calpain activity is enhanced in nigral dopamine neurons of postmortem tissues from human PD cases. These findings support a novel role for calpains in the molecular events related to nigral neuron dysfunction in PD.

Materials and Methods

Mice

All procedures involving animals were approved by the University of Ottawa Animal Care Committee and maintained in strict accordance with the *Guidelines for the Use and Treatment of Animals* put forth by the Animal Care Council of Canada and endorsed by the Canadian Institutes of Health Research.

MPTP

N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (25 mg/kg, i.p., measured as free base; MPTP-HCl; Sigma, St. Louis, MO) was administered to male C57BL/6 mice (8–10 weeks old; Charles River Laboratories, St. Constant, Quebec) once a day for 5 consecutive days (Tatton and Kish, 1997; Xia et al., 2001) (Fig. 1*A*). Mice used as controls received an equivalent volume of saline (0.9%) once daily.

Calpain inhibition

Osmotic minipumps (Alza, Palo Alto, CA; model 1007D) were implanted into the right lateral ventricle 1.0 mm rostral and 2.2 mm to the right of bregma with the cannula extending to a depth of 2.5 mm from the skull surface. Pumps were prefilled with 200 μl of either vehicle (Krebs'-Ringer's solution) or MDL-28170 (160 μM; carbobenzylzoxy-Val-Phe-H) and implanted 24 hr before the start of the MPTP dosing regimen (Fig. 1*B*). MDL-28170 was diluted in vehicle containing 10% cycylodextrins (RBI, Natick, MA) from a 20 mM stock solution (in dimethyl sulfoxide). Behavioral analyses and assessment of nigral dopamine neuron survival in all osmotic pump-implanted mice was performed 2 or 3 weeks after the initiation of MPTP.

MPP+ measurement

Twenty-four hours after the implantation of osmotic pumps delivering either MDL-28170 or vehicle (see above), striatal concentrations of MPP+ were measured 90 min after a single injection of MPTP. HPLC measurements were performed as described previously (Przedborski et al., 1996).

Adenoviral gene delivery

Additional groups of mice were administered recombinant adenoviruses that expressed either the calpain inhibitor protein calpastatin or the bacterial reporter gene *lacZ*. Calpastatin (RNCAST104) (Melloni et al., 1998) was excised from pGEX 6P1 using *Bam*H1 and *Eco*R1 and ligated into the pAd-lox vector for amplification into recombinant adenovirus, as described (Hardy et al., 1997). Adenoviruses (3 μl; 1×10^7 particles μl^{-1} per construct) expressing either calpastatin (Ad.CALP) or a control expression marker, lacZ (Ad.lacZ), were stereotaxically injected into the right dorsolateral striatum (0.5 mm rostral and 2.2 mm to the right of bregma and 3.4 mm below the skull surface) at an infusion rate of 0.5 μl/min using a syringe pump (PHD2000, Harvard Apparatus, St. Laurent, Quebec). Mice were challenged with MPTP 1 week after adenovirus injection (Fig. 1*C*) to permit sufficient time for retrograde transport and expression of the adenovirus-derived proteins. Assessment of dopamine neuron survival was made 2 weeks after the start of the MPTP dosing regimen.

Immunohistochemistry

Brain tissues from mice injected with MPTP or saline were collected for immunohistochemical analyses as described previously (Crocker et al., 2001a,b). Antibodies used were tyrosine hydroxylase (TH) (1:1000; IncStar), dopamine transporter (DAT) (1:5000; Chemicon), and calpastatin [1:500 (Melloni et al., 1998)]. Calpain activity was detected using an antibody generated against a peptide fragment of α-spectrin derived by calpain-mediated cleavage (38-4, 1:2000), which is highly selective and specific for calpain-cleaved fragments of α-spectrin but not for α-spectrin cleavage by other proteases (Roberts-Lewis et al., 1994). Immunoreactivity was visualized using an avidin–biotin complex peroxidase reaction (Crocker et al., 1998). Immunofluorescent labeling was visualized using secondary antibodies (Jackson Labs): anti-mouse IgG-conjugated CY3 antibody for detection of TH (Jackson Labs) and anti-rabbit IgG or anti-goat IgG conjugated to FITC for detection of 38-4 and calpastatin, respectively.

Assessment of neuronal loss

Loss of neurons in the SNc was determined by serial section analysis of the total number of TH-positive (TH+) neurons. Every sixth coronal section throughout the entire rostral-caudal (RC) axis of the murine SNc was collected for assessment of neuronal survival by immunohistochemistry (Franklin and Paxinos, 1997). Adjacent SNc tissue sections from each animal were also stained with cresyl violet to validate immunohistochemical determination of nigral neuron survival. Estimates of total TH+ and cresyl-stained nigral neuron populations were calculated using Abercrombie's correction (Abercrombie, 1946).

For those experiments in which adenoviruses were used, only the sections within the range of the medial terminal nucleus (MTN) were evaluated, because intrastriatal administration results in retrograde labeling of only a subpopulation of SNc neurons (Crocker et al., 2001a). The total numbers of TH+ neurons (−3.08 to −3.28 mm) in the ipsilateral and contralateral hemispheres were counted separately from at least six sections for each animal. Treatment groups were averaged and differences were analyzed by a one-way ANOVA, followed by Newman–Keuls test, and considered significant when $p < 0.05$.

Table 1. Age and postmortem interval (PMI) of matched human nigral tissue samples

Sample number	Disease condition	Age	PMI (hr)
1	Parkinson's disease	63	2.7
2	Parkinson's disease	83	5.5
3	Parkinson's disease	73	8.2
4	Parkinson's disease	78	13.5
		Average 74.3	Average 7.5
5	Control	85	4
6	Control	76	5.4
7	Control	68	14.8
8	Control	65	17.3
9	Control	70	18
		Average 72.8	Average 11.9

Table 2. Summary of clinical case histories of Parkinson's disease cases used in our study based on available records from Harvard Brain Bank

Case number	Age of diagnosis	Duration of illness	Medications	Cause of death
1	59	4 years	a,b,c,d,e,f	Cardiopulmonary arrest
2	68	15 years	a,h,n,p,q,r,s,t,u,v w,x,y,z,aa,bb,cc,dd ee,ff,gg	Congestive heart failure
3	45	28 years	c,g,h,l,j,k,l,m	Dehydration
4	66	12 years	a,n,o	Epidural hematoma

Medications: a, Sinemet; b, Propanolol; c, Parlodel; d, Eldepryl; e, Prozac; f, Diphenhydramine; g, L-Dopa; h, Zantac; i, Ditropan; j, Symmetrel; k, Xanax; l, Halclon; m, Deprenyl; n, Permax; o, Docusate calcium; p, Amitripyline; q, Ativan; r, Bactroban; s, Blephamide; t, Opthalmic cormax; u, Dicloxacillin; v, Digoxin; w, Fentany; x, Furosemide; y, Ibuprofen; z, Lanoxin; aa, Lasix; bb, morphine; cc, Olanzepine; dd, Prednisone; ee, Salicyclic acid; ff, Vasotec; gg, Zaroxlolyn.

Striatal densitometry

Quantification of striatal dopaminergic fibrous staining and striatal FosB-positive nuclei was performed on striatal tissues from animals 14 d after the start of MPTP. Counts were made by sampling an area 660 × 800 μm in at least five sections per animal using computer-assisted image analysis software (Northern Eclipse, Empix Imaging, Mississauga, ON), as described previously (Crocker et al., 2001a,b). Analyses were performed by an individual unaware of the experimental treatments.

Striatal HPLC

Levels of neurotransmitters and metabolites were separated and measured simultaneously from single perchloric acid (PCA) extracts using HPLC with electrochemical detection as described previously (Hayley et al., 1999; Crocker et al., 2001a). Extracts were taken from groups of mice 2 weeks after the start of MPTP injections.

Behavioral analyses

Behavioral analyses were performed to assess locomotor function 14 d after the start of MPTP dosing (Fig. 1*B*).

Novel environment. Walled 32- × 32-inch-square arenas were used for open field testing of mice, using a video camera and analysis software (Videomex 5, Columbus Instruments). Total horizontal ambulatory distance traveled during the 1 hr observation period was reported in centimeters. Additional groups of unlesioned and MPTP-lesioned (VEH-MPTP) mice were also administered L-Dopa (15 mg/kg, i.p.) to determine whether the locomotor deficit in this novel environment was reversible with dopamine replacement.

Home cage. Additional groups of animals were analyzed for behavioral performance in response to amphetamine administration (2.0 mg/kg, s.c.) using activity cages outfitted with infrared detector arrays, as described previously (Merali and Piggins, 1990). Home cages were used for amphetamine (2.0 mg/kg)-induced activity to exclude the influence of a novel environment on behavioral performance. All assessments of behavioral performance were performed by individuals blinded to the experimental treatments.

Striatal neurotensin radioimmunoassay

Using a modification of the method of Palkovits (Palkovits and Brownstein, 1988), serial coronal cryostat sections were used to micropunch (Micro Punch MP-600, ASI Instruments) striatal samples from tissues from each subject of each treatment group. Detection and quantification of neurotensin (NT) were achieved through a high-sensitivity double-antibody liquid-phase RIA kit obtained through Phoenix Pharmaceuticals (Belmont, CA). A four-parameter logistic curve fit model was used for interpolation of the standard curves. Sensitivity of the assay had an IC_{50} = 19.1 pg per tube. Tissues were extracted from mice 14 d after the start of MPTP (n = 7–8 per group).

Human brain samples

Paraffin-embedded postmortem human tissues from Parkinson's disease brains and control brains were provided by the Harvard Brain Tissue Resource Center (HBTRC) and were for analysis of calpain-related gene expression (Table 1). The age and postmortem interval from matched samples did not differ ($p < 0.25$). Diagnoses were made on the basis of medical histories and postmortem confirmation by HBTRC. Of the PD subjects, the average age of symptom onset was 52.8 ± 5.8 years, whereas the average duration of illness was 15.6 ± 3.9 years. Pharmacotherapy for all cases included L-Dopa or Sinemet, and Eldepryl (Table 2).

Because the absolute number of melanized dopamine neurons varies between each individual, the number of immunopositive neurons was calculated as a proportion of the number of pigmented neurons for each section and subject ($F = 55.95$; $p < 0.0001$). Hence, determinations of significance between the estimated proportion of immunopositive dopamine neurons in the PD subjects were compared with the control cases using a one-tailed Mann–Whitney rank sum test (Hirsch et al., 1988; Hartmann et al., 2001). Differences were considered significant when $p < 0.05$.

Results

MPTP evokes sustained activation of calpains in the SNc

We first determined whether calpains may be activated in SNc neurons after chronic MPTP treatment in mice. Using an antibody that selectively recognizes an epitope within α–spectrin that has been exposed by calpain proteolysis (neoepitope) (Roberts-Lewis et al., 1994), midbrain sections from mice treated with MPTP were analyzed for evidence of increased calpain-mediated proteolysis by immunohistochemistry. Mice that were treated with saline did not exhibit any detectable increase in neoepitope labeling (Fig. 2*a*), whereas a noticeable and sustained increase in calpain-mediated proteolysis was observed in the nigral region of mice that had been treated with MPTP (Fig. 2*b*,*c*). Closer examination of this region revealed that the pattern of calpain-cleaved α–spectrin was located predominantly in the cytoplasm and was punctate in appearance (Fig. 2*e*,*f*). This punctate appearance resembled calpain translocation to intracellular membrane compartments in hippocampal neurons after ischemia *in vivo* (Yamashima et al., 1998). Intracerebroventricular infusion of the pharmacological calpain inhibitor MDL-28170 prevented the emergence of immunoreactivity for calpain-cleaved α-spectrin after chronic MPTP administration (Fig. 2*d*).

To demonstrate that the observed increase in calpain-related activity indeed was in nigral dopamine neurons, we performed immunofluorescent double-labeling experiments using TH as the dopaminergic marker. In midbrain tissues from untreated mouse brains, calpain-cleaved α–spectrin immunostaining was weak and diffusely cytoplasmic in nigral dopaminergic neurons (Fig. 2*g*,*i*). However, in tissues from mice treated with MPTP, this cytoplasmic immunolocalization within dopamine neurons exhibited a bright speckled appearance that became progressively more intense by 14 d (Fig. 2*h*,*j*) after cessation of MPTP administration.

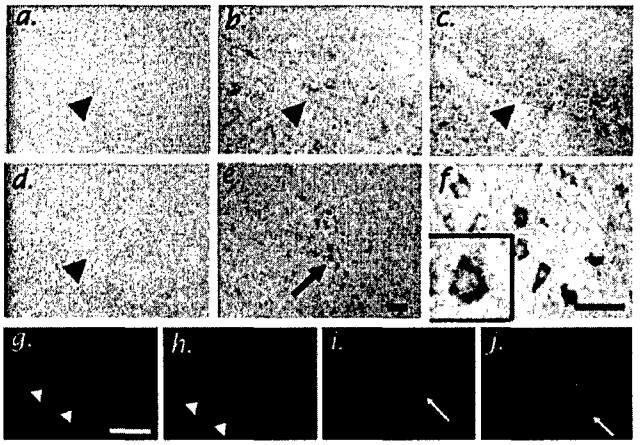

Figure 2. Increased calpain proteolysis in nigral dopamine neurons of MPTP-treated mice. Detection of calpain-cleaved α-spectrin was absent in the SNc of saline-treated mice (*a*). Administration of MPTP (25 mg/kg measured as free base, i.p.) daily for 5 consecutive days resulted in increased reactivity for calpain-cleaved α-spectrin 7 d (*b*) and 14 d (*c*) later. Intracerebroventricular coadministration of the calpain inhibitor MDL-28170 blocked MPTP-induced calpain-mediated cleavage of α-spectrin 14 d after MPTP (*d*). MPTP-treated mice that were coadministered the vehicle (*e*, *f*) also revealed a predominance of punctate and cytoplasmic patterns of immunostaining for calpain-cleaved α-spectrin, whereas nuclear immunostaining was weak and not pronounced (*f*, inset). Immunohistofluorescence confirmed the increased expression of calpain-cleaved α-spectrin (*g*, *i*) in tyrosine hydroxylase-immunoreactive cell bodies in the substantia nigra (*h*, *j*) of MPTP-treated mouse brain tissues 14 d (*i*, *j*) after treatment, but not in the SNc of saline-treated mice (*g*, *h*). Clusters of speckled immunoreactivity were nonreactive for glial acidic fibrillary protein (data not shown). Arrowheads indicate region of the SNc (*a–d*), and arrow in *e* indicates area magnified in cell inset (*f*). Scale bars: *a–e*, 100 μm; *g–j*, 15 μm; *f*, inset, 25 μm.

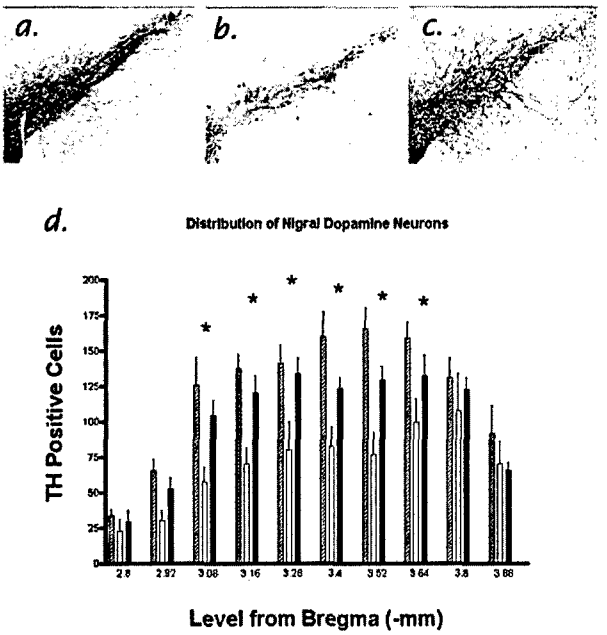

Figure 3. Protection of nigral dopamine neurons by calpain inhibition. Representative coronal midbrain sections (at level −3.52 mm caudal to bregma) from mice 2 weeks after treatment with saline (*a*), MPTP with vehicle (*b*), or MPTP with the calpain inhibitor MDL-28170 (*c*). *d*, Quantitative comparison of nigral dopamine (TH+) neuron survival in mice treated with saline (hatched bars), vehicle and MPTP (white bars), or MDL 28170 and MPTP (black bars). Bars represent mean number (±SEM) of neurons according to the rostrocaudal distribution measured (millimeters) from bregma (Franklin and Paxinos, 1997) from eight mice per group (ANOVA, $p < 0.01$; Newman–Keuls, $p < 0.05$).

Calpain inhibition prevents nigral dopamine neuron degeneration

To examine whether the increased calpain-mediated proteolytic activity was related to the loss of dopamine neurons after MPTP administration, we infused mice with either the calpain inhibitor (MDL-28170) or its vehicle (VEH) and then treated them with MPTP. This pharmacological calpain inhibitor has been previously reported to attenuate neuronal loss after transient ischemia (Markgraf et al., 1998). Exposure of mice to MPTP produced a substantial loss of nigral dopamine neurons (Fig. 3*b*) when compared with unlesioned controls (Fig. 3*a*, Table 3). However, intracerebroventricular administration of the calpain inhibitor MDL-28170 significantly attenuated the loss of nigral dopamine neurons 2 weeks after the initiation of MPTP treatment (Fig. 3*c*). Analysis of the distribution of nigral dopamine (TH+) neurons along the rostrocaudal axis revealed that the dopamine neurons within the medial aspect of the SNc were the most sensitive to MPTP-induced loss. Nigral neuron survival was also compared using Abercrombie's correction for estimation of neuronal populations (Abercrombie, 1946). By this method, inhibition of calpains using MDL-28170 (MDL-MPTP) significantly attenuated the overall loss of nigral TH+ neurons to 17.8% versus a 55.9% loss of nigral dopamine neurons after MPTP treatment in vehicle-treated controls (VEH-MPTP). One-way ANOVA revealed that the neuroprotection conferred by calpain inhibition did not differ from saline-treated controls at 2 weeks after MPTP ($p < 0.0008$; Newman–Keuls, $p < 0.05$) (Fig. 3*d*, Table 3). To evaluate whether inhibition of calpains was a transitory effect on neuron survival after MPTP treatment, we next examined the degree of nigral neuron survival 3 weeks (21 d) after MPTP in mice coadministered vehicle or the calpain inhibitor. Consistent with the neuroprotection observed at 14 d, at 21 d after MPTP significantly greater numbers of nigral dopamine neurons were still evident, as evaluated by either TH immunostaining or cresyl violet staining (data not shown), in the MPTP-lesioned mice treated with the calpain inhibitor MDL-28170 when compared with vehicle-lesioned controls (Table 3).

MPTP metabolism is not affected by calpain inhibition (MDL-28170)

The neurotoxic effects of MPTP on dopamine neurons require the metabolism of MPTP to MPP+ by monoamine oxidase (Heikkila et al., 1984; Javitch et al., 1985; Przedborski et al., 2001). Hence, to determine whether pharmacological inhibition of calpains modified the metabolism of MPTP (and thus indirectly prevented nigral degeneration), we extracted striatal tissues from mice 90 min after a single injection of MPTP (30 mg/kg) and measured levels of MPP+. This analysis revealed that calpain inhibition did not influence the metabolism of MPTP to MPP+ *in vivo* ($p > 0.28$) (Table 3), indicating that the observed protection by calpain inhibition was not caused by an indirect mitigation of injury through impaired MPTP metabolism.

Calpastatin overexpression is neuroprotective

Next, we engineered a recombinant adenovirus to overexpress the endogenous calpain inhibitor protein, calpastatin, *in vivo* (De Tullio et al., 1998; Crocker et al., 2001a). Adenoviruses expressing either lacZ (Ad.lacZ) or calpastatin (Ad.CALP) were injected directly into the right dorsolateral striata of mice to produce retrograde transport (Fig. 4*b*) and expression within nigrostriatal dopamine neurons (Fig. 4*d*) (Ridoux et al., 1994; Crocker et al., 2001a). The degree of neuronal loss in the contralateral (non-virus injected) hemispheres of Ad.lacZ and Ad.CALP mice did

Table 3. Calculation of neuronal MPP+ metabolism (nanograms per milligram tissue) and calculations of nigral dopamine neuron (SNc) survival in saline and MPTP treatment groups

	Saline	VEH-MPTP	MDL-MPTP		*p*
MPP+	N/A	4.566 ± 0.579	4.064 ± 0.644	(*t*)	0.5781
14 d TH	12259 ± 837.8	6854 ± 859.5*	10069 ± 629.6	(A)	0.0008
21 d TH	14082 ± 2123.8	5061 ± 993.94*	12905 ± 1789.9	(A)	0.0038
21 d CV	17086 ± 881.8	7366 ± 1923.2*	13961 ± 1164.3	(A)	0.0003

Estimates of nigral neuron populations were performed on tyrosine hydroxylase (TH)- or cresyl violet (CV)-stained midbrain tissue sections and calculated using Abercrombie correction. Data are presented as group mean ± SEM. Statistical differences are given as *p* values for either *t* test (*t*) or one-way ANOVA (A) with significance represented by asterisks.

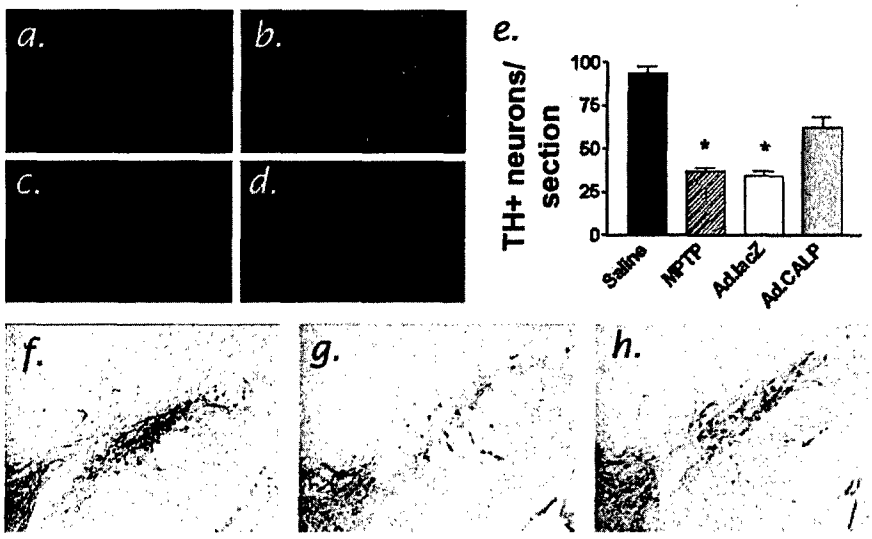

Figure 4. Increased calpastatin in the SNc ipsilateral to intrastriatal adenovirus administration (*a*), when compared with the contralateral hemisphere (*b*), and colocalization within dopaminergic SNc neurons (*c*, *d*). Representative coronal midbrain section at the level of the MTN (−3.16 mm caudal to bregma) (Franklin and Paxinos, 1997) from a saline-treated mouse (*f*) and the ipsilateral hemisphere of MPTP-treated mice that received intrastriatal administration of recombinant adenoviruses containing either lacZ (*g*) or the calpain inhibitor protein calpastatin (*h*). Images are representative of dopamine neuron survival observed 2 weeks after treatment with MPTP ($n = 6$ per group). *e*, Quantification of dopamine neuron survival per section in the ipsilateral SNc of Ad.lacZ, Ad.CALP, and uninjected-MPTP-treated or unlesioned mice. *ANOVA, $p < 0.0001$; Newman–Keuls, $p < 0.001$; Ad.CALP versus Ad.lacZ or MPTP.

not differ after MPTP treatment (*t* test; $p > 0.84$). Compared with the SNc of unlesioned mice (Fig. 4*e*), the number of surviving dopamine neurons in the ipsilateral hemisphere of MPTP-treated Ad.lacZ-treated mice was significantly less and comparable with the loss of dopamine neurons observed in the contralateral (non-virus injected) SNc (Fig. 4*f*,*h*). In contrast, the number of nigral neurons in the ipsilateral (adenoviral injected) SNc of Ad.CALP-treated mice was significantly greater than its contralateral hemisphere (Fig. 4*g*,*h*). Quantitative comparison indicated that adenoviral expression of calpastatin significantly attenuated MPTP-induced neuron loss, doubling the number of surviving TH+ SNc neurons (Fig. 4*h*). To verify that adenovirus-mediated expression of calpastatin was affecting calpain-related proteolysis after MPTP administration, we also determined that calpain-cleaved α-spectrin (38-4) immunoreactivity was significantly attenuated in the ipsilateral calpastatin-expressing SNc when compared with the control contralateral side (data not shown).

MPTP-induced hypolocomotion is abrogated by calpain inhibition

The ultimate therapeutic goal of neuroprotection is to ameliorate functional impairment. Accordingly, we next determined whether the protection of nigral dopamine neurons by inhibition of calpain-mediated proteolysis was also accompanied by an absence of locomotor deficit, one important hallmark of PD. Using a video-tracking system, we evaluated the horizontal locomotor activity of mice in a novel environment. In this setting, MPTP-treated mice displayed a modest 30% decrease in spontaneous motor activity when compared with either group of non-MPTP-treated control animals (Fig. 5*a*). In contrast, mice that received the calpain inhibitor MDL-28170 exhibited significantly more activity than MPTP-lesioned controls ($p < 0.01$) and did not differ in total spontaneous activity from unlesioned controls (Fig. 5*a*) ($p > 0.05$). The deficit in spontaneous locomotor activity observed in MPTP lesioned mice in this open field test was also reversed by administration of L-Dopa (15 mg/kg, i.p.) at the time of testing (Newman–Keuls; $p > 0.05$). Thus, MPTP treatment produced a significant reduction in spontaneous locomotor performance that was prevented by inhibition of calpains (ANOVA, $p < 0.008$; $p > 0.05$ vs saline or VEH-MPTP + L-Dopa). Mice treated with calpain inhibitor but without MPTP did not differ from saline-treated mice (data not shown).

Next we examined whether the improved activity in calpain-treated mice was a consequence of enhanced dopamine-related neurotransmission. To test this, additional groups of mice were treated with saline, MPTP and vehicle, or MPTP and MDL-28170. These groups were kept in their home cages to minimize any influence of natural exploratory behaviors observed in a novel environment. These cohorts were challenged with amphetamine (2.0 mg/kg, i.p.) to stimulate the release of dopamine from intact nerve terminals. In this experiment, amphetamine-induced hyperlocomotor activity was used as a functional measure of dopamine-related function within the basal ganglia. Amphetamine-induced locomotor responses were severely impaired in MPTP-treated mice that were infused with the vehicle solution (Fig. 5*b*, VEH-MPTP), whereas MPTP-treated mice that were cotreated with calpain inhibitor displayed robust activity in response to amphetamine challenge (MDL-MPTP) and exhibited a level of activity equal to saline-treated control animals (Fig. 5*b*). Taken together, these results indicate that calpain inhibition prevented impaired locomotor performance in response to either a novel environment or an amphetamine challenge. Interestingly, there is some variation in the literature regarding the presence and severity of motor behavior deficits in the MPTP mouse model, perhaps because of subtle differences in mouse stains, dosing regimens, or modes of analyses (Sedelis et al., 2001). How-

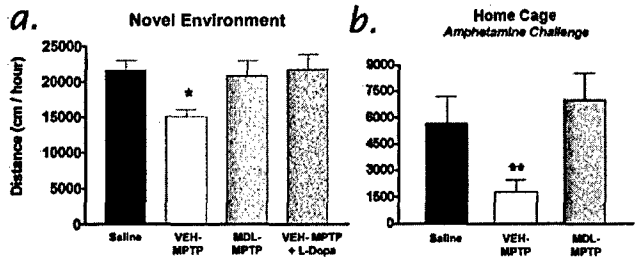

Figure 5. The neuroprotective effects of calpain inhibition on MPTP-induced toxicity in mice is associated with normalized locomotor behaviors. Two weeks after saline or MPTP treatment, groups of mice were assessed for spontaneous motor activity in a novel environment (open field) for a 60 min period, as described in Materials and Methods (*a*). Horizontal locomotor activity was reported as distance traveled (mean ± SEM; *ANOVA, $p < 0.001$; Newman–Keuls, $p < 0.01$; either group vs VEH-MPTP). Additional groups of mice were administered amphetamine (2.0 mg/kg, i.p.), and hyperactivity was measured in their home cages using beam-break activity monitors (*b*). Total activity over 30 min is plotted as mean ± SEM. Data represent $n =$ 6–9 per group per treatment for *a* and *b*.

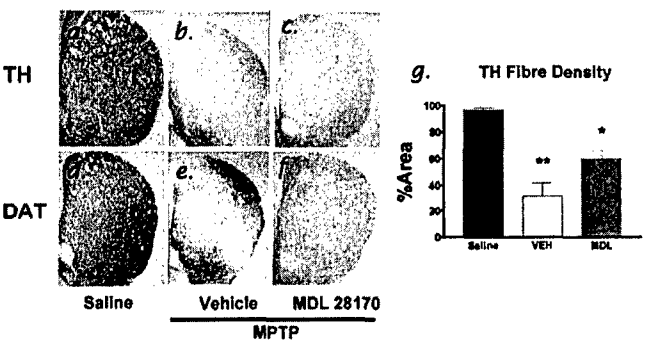

Figure 6. Calpain inhibition does not prevent striatal denervation. Representative immunohistochemical detection of tyrosine hydroxylase (TH) (*a–c*) and dopamine transporter (DAT) (*d–f*) in striatal sections from mice treated with saline (*a*, *d*), MPTP, and vehicle (*b*, *e*), or MDL-28170 and MPTP (*c*, *f*). *g*, Quantification of striatal TH fiber densities (ANOVA, $p < 0.001$; Newman–Keuls, $^{**}p < 0.001$, and $^{*}p < 0.05$, vs saline).

ever, our findings are consistent with the growing majority of studies reporting hypolocomotion.

Calpain inhibition and striatal denervation

Because calpain inhibition prevented the detrimental effects of MPTP on locomotor performance, we next evaluated the status of nigrostriatal function in groups of mice by assessing the expression of various neurochemical and immunohistochemical markers for dopaminergic neurotransmission. Immunohistochemical detection of TH fiber staining in striatal tissue sections from saline-treated (Fig. 6*a*), VEH-MPTP-treated (Fig. 6*b*), or MDL-MPTP-treated (Fig. 6*c*) mice revealed that MPTP-induced depletion of nigrostriatal dopaminergic fibers was only slightly mitigated by treatment with the calpain inhibitor. Densitometric analysis of striatal TH staining revealed a significant loss of TH fibers in VEH-MPTP-treated mice and a lessened but significant loss in mice treated with calpain inhibitor (Fig. 6*g*). Although calpain inhibition partially ameliorated the degree of loss of TH fibrous staining, both groups of MPTP-treated mice were significantly different from saline-treated controls. Finally, adjacent tissues sections assessed for expression of DAT revealed a similar degree of dopamine terminal loss as that detected by TH immunostaining in these groups (Fig. 6*d–f*).

To establish whether the modest DA terminal protection offered by calpain inhibition correlated with dopamine levels in the

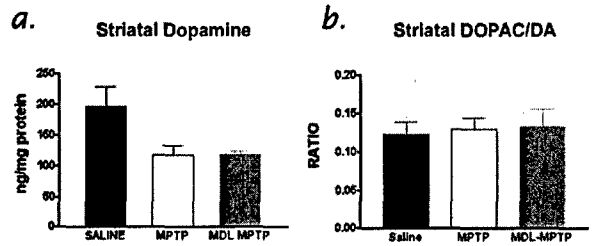

Figure 7. HPLC analysis of striatal dopamine (*a*) and DOPAC concentrations (*b*) shows no preservation of striatal dopamine or metabolism after MPTP administration in either vehicle (VEH-MPTP) or calpain inhibitor-treated (MDL-MPTP) mice. Data represent mean ± SEM ($n =$ 6–8 per group). Where no significant differences existed between VEH-MPTP and MPTP treatment groups, data were pooled as "MPTP."

striatum, HPLC detection of DA and metabolites was performed on tissues VEH-MPTP, MDL-MPTP, and saline-treated mice. This analysis revealed that MPTP administration caused a significant 60.25% (average) drop in striatal dopamine levels in all MPTP-treatment groups, including those receiving the calpain inhibitor (Fig. 7*a*) ($p < 0.0002$). Analyses of the dopamine metabolites, DOPAC and homovanillic acid, did not indicate any compensatory increase in dopamine turnover (Fig. 7*b*), which has been reported by some (Vila et al., 2001) but not by others (Yang et al., 1998; Eberhardt et al., 2000). Nevertheless, in this study the absence of protection or compensation of dopamine levels in the striata of calpain inhibitor-treated mice therefore cannot account for the improved behavioral performance in this treatment group (Fig. 7*b*).

Calpain inhibition prevents postsynaptic changes in gene expression in the striatum

Because calpain inhibition prevented the loss of motor function in MPTP lesioned mice but did not prevent the loss of striatal dopaminergic fibers, we surmised that one possible consequence of nigral neuroprotection by calpain inhibition might have been the indirect modulation of postsynaptic changes in the denervated striatum. To analyze for this possibility, we looked for changes in postsynaptic markers of denervation in the striatum of mice. The first marker of neuronal plasticity that is upregulated substantially in the striatum in response to dopaminergic denervation is the immediate early gene, FosB (Doucet et al., 1996; Perez-Otano et al., 1998). FosB has garnered attention recently for its roles in the processes mediating the neural plasticity associated with addiction (Nestler et al., 1999) and as a causal signal mediating the supersensitivity of striatal dopamine receptors after dopaminergic denervation (Crocker et al., 1998; Andersson et al., 1999). Striatal tissue sections from mice treated with saline, MPTP, VEH-MPTP, or MDL-MPTP were processed for immunohistochemical detection of FosB. Consistent with previous reports (Doucet et al., 1996; Perez-Otano et al., 1998), MPTP induced a profound increase in FosB expression in the striatum (Fig. 8*a–c*). However, coadministration of MDL-28170 significantly attenuated the MPTP-induced increase in striatal FosB expression (Fig. 8*d*). Quantification of striatal FosB nuclei showed that mice lesioned with MPTP and treated with the calpain inhibitor expressed significantly fewer FosB-like immunoreactive neurons than other MPTP-lesioned animals (Fig. 8*e*). Interestingly, FosB expression in MDL-MPTP-treated mice did not differ from saline-treated controls ($p > 0.05$).

The role of neuropeptides as modulators of basal ganglia activity has led to the suggestion that these molecules may contrib-

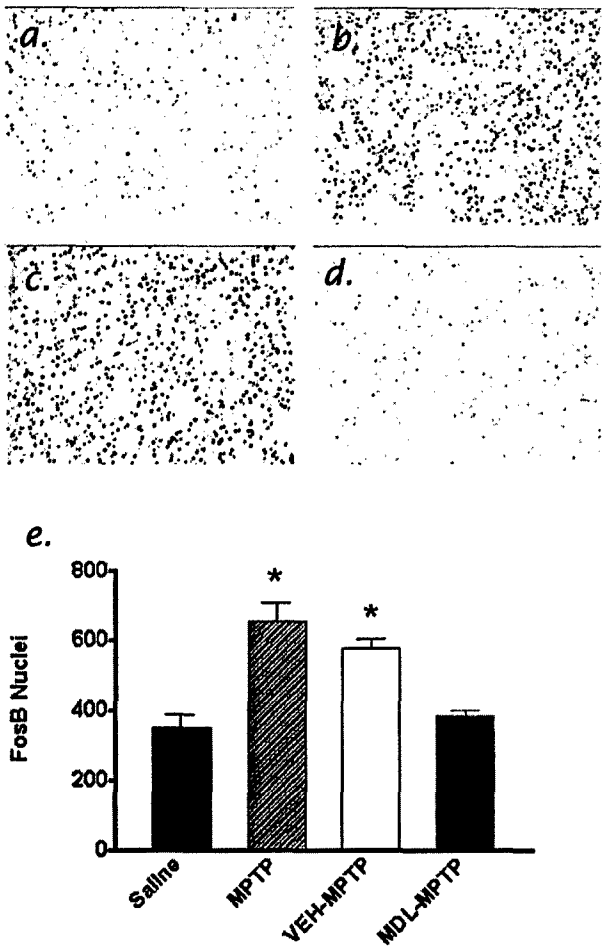

Figure 8. MPTP-induced increased striatal FosB expression is attenuated by calpain administration. Representative coronal forebrain sections depict low basal expression of FosB in the striatum (*a*) and a dramatic elevation 2 weeks after chronic administration of MPTP (*b*). Striatal sections from mice administered MPTP and vehicle revealed a comparable upregulation of FosB (*c*), whereas tissues from mice administered MPTP and the calpain inhibitor MDL-28170 revealed an attenuation of postsynaptic FosB expression (*d*). *e*, Quantification of striatal FosB-positive nuclei show significant differences between treatment groups (*ANOVA, $p < 0.0005$; Newman–Keuls, $p < 0.01$ compared with saline control). FosB expression in MDL-MPTP-treated mice did not differ from saline-treated controls ($p > 0.05$). Data represent mean ± SEM ($n = 5–6$ per group).

ute to dysfunction related to PD pathology. As a second marker of postsynaptic changes resulting from dopaminergic denervation, we looked at NT because recent reports suggest that endogenous NT may antagonize dopamine neurotransmission (Ford and Marsden, 1990; Radke et al., 1998; Binder et al., 2001), and striatal expression of NT has been reported to increase after MPTP administration to mice (Martorana et al., 2001) and in postmortem human PD tissues (Fernandez et al., 1995; Schimpff et al., 2001). Given the possible modulatory effects that NT may exert on basal ganglia function, we therefore determined whether MPTP-induced changes in neurotensin were modified by administration of the calpain inhibitor MDL-28170. Radioimmunoassay of striatal tissues from saline- and MPTP-treated groups of mice revealed a 2.5-fold increase in neurotensin levels in MPTP-treated mice 2 weeks after cessation of treatment, when compared with saline-treated controls (Fig. 9). In contrast, MPTP mice treated with the calpain inhibitor did not exhibit any changes in striatal NT expression (Fig. 9), which suggests that calpain inhibition

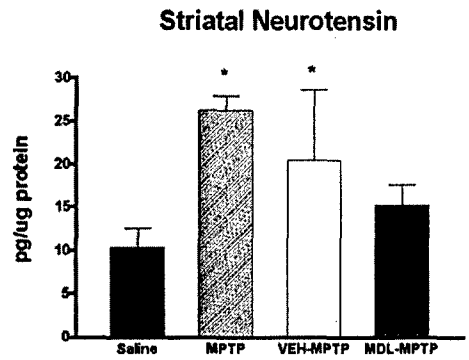

Figure 9. Radioimmunoassay of striatal neurotensin revealed increased expression after treatment with MPTP that was attenuated by calpain inhibitor treatment (*ANOVA; $p < 0.05$). Data represent mean ± SEM ($n = 7–8$ per group).

prevented postsynaptic changes in markers of gene and neuropeptide changes in the dopaminergic denervated striatum.

Evidence for calpain activation in postmortem human Parkinson's disease tissues

Finally, we examined whether calpain activation participates in the human PD condition, as observed in the MPTP-treated mice. Tissues from the midbrains of individual cases with premortem diagnosis of either Parkinson's disease or no known neurological condition were analyzed for immunohistochemical detection of calpain activity using the calpain-cleaved α–spectrin antibody (Tables 1, 2). Qualitatively, the distribution of calpain-cleaved α–spectrin in nigral neurons of control cases was weak and diffusely cytoplasmic, whereas tissues from PD subjects exhibited an intense speckled appearance that resembled the staining observed in the nigral dopamine neurons of MPTP-treated mice (Figs. 10 and 2, respectively). The proportion of pigmented SNc neurons immunopositive for calpain-cleaved α–spectrin was significantly higher in PD cases (52.83%) when compared with expression in control subjects (9.2%; Mann–Whitney; $p = 0.0079$) (Fig. 10*D*). These data provide a potentially interesting and novel correlation of the human PD condition with changes observed in MPTP-treated mice.

Discussion

Although calpains mediate various cellular functions, the results of this study support the hypothesis that inappropriate or sustained activation of calpain-related proteolysis may precipitate neuropathology (Saito et al., 1993; Roberts-Lewis et al., 1994; Mouatt-Prigent et al., 1996; Markgraf et al., 1998; Lee et al., 2000). In this regard, it is important to note that calpains are calcium dependent, indicating that processes involving the management of intracellular calcium can influence the state of calpain activation. Maintenance of calcium homeostasis is critical for neuronal viability, necessitating tight regulation of its intracellular concentrations. In neurons, mitochondria are a central store of calcium as well as the primary source of cellular oxidative metabolism. The finding that dopaminergic neurotoxins such as MPTP (MPP+) are also mitochondrial toxins has led to the hypothesis that impaired mitochondrial function may underlie the nigral pathogenesis of PD. Indeed, deficits in complex 1 activity in PD patients (Greenamyre et al., 2001) and evidence for increased oxidative stress in nigral neurons have been reported (for review, see Przedborski and Jackson-Lewis, 2000). In addition, mitochondria are an integral participant in signaling pathways of apoptotic neuronal death, which has been proposed to mediate

neuronal loss in PD (Cassarino et al., 1999; Hartmann et al., 2000; Mochizuki et al., 2001; Vila et al., 2001; Viswanath et al., 2001).

Several lines of evidence support the hypothesis of calcium-mediated pathology in PD: (1) MPP+ evokes sustained increases of intracellular calcium in neurons (Frei and Richter, 1986; Chen et al., 1995; Sherer et al., 2001); (2) mitochondria from PD patients exhibit a diminished capacity to sequester calcium (Sheehan et al., 1997); (3) nigral neurons that express the calcium-binding protein, calbindin D, are less vulnerable to degeneration in PD, perhaps because of the ability to buffer increased levels of intracellular calcium (Yamada et al., 1990; Lavoie and Parent, 1991; Hirsch et al., 1997; McMahon et al., 1998; Damier et al., 1999); (4) calcium-regulating genes, such as nitric oxide synthase, have been implicated in dopamine neuron degeneration in PD (Schulz et al., 1995; Liberatore et al., 1999); and (5) energy disruption alone is insufficient to explain the toxicity of MPP+ (Nakamura et al., 2000). It is important to note that although the sustained calcium-mediated activation of calpains in mice in our study is likely a consequence of the mitochondrial toxicity of MPTP, in the human cases of PD, calcium may arise from mitochondria or other possible sites. For instance, mitochondrial dysfunction results in a paucity of energy (ATP) that could evoke release from endoplasmic reticular stores (Mattson et al., 2000; Paschen and Frandsen, 2001) or modulate influx of calcium from extracellular sources (Greenamyre et al., 1999). Finally, we cannot rule out the possibility that calpain regulation may occur through mechanisms other than an abberant calcium response. This is important because the physiological regulation of calpains is complex, involving numerous factors including calcium, phosphorylation, and nitrosylation (Sato and Kawashima, 2001; Shiraha et al., 2002; Forsythe and Befus, 2003). Although our results validate a role for calpains in PD, the identity of the essential calpain species involved in the loss of nigral dopamine neurons after MPTP is also unresolved. It is important to note that although previous work on postmortem human tissues has reported increased expression of m-calpain in PD, it not presently clear whether MPTP also recapitulates this selectivity of calpain involvement in mice.

The manner by which calpains mediate dopaminergic death remains to be elucidated. Putative calpain substrates that have been associated with neurodegeneration in PD include c-Jun (Hirai et al., 1991; Saporito et al., 1999; Xia et al., 2001) and p53 (Trimmer et al., 1996; Gonen et al., 1997). In general, the short half-life of calpain substrates coupled with the restricted nature of calpain cleavage of these target proteins has led to the suggestion that calpains act to modify rather than completely catabolize substrate proteins (Suzuki et al., 1992; Carafoli and Molinari, 1998). For instance, the transcriptional activity of c-Jun is significantly reduced in the presence of activated calpain, suggesting that calpains may influence signal transduction by modulating gene expression (Suzuki et al., 1992). In addition, the cyclin-dependent kinase 5 (cdk5) activating protein p35 has been reported to be cleaved by calpains into a p25 isoform that results in unregulated cdk5 activity and neurodegeneration (Patrick et al., 1999; Lee et al., 2000). Hence, various potential calpain substrates may directly or indirectly contribute to calpain-related degeneration of dopamine neurons.

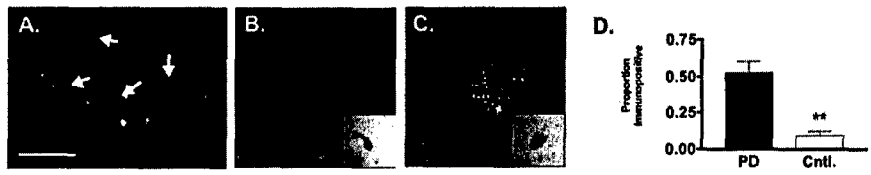

Figure 10. Immunohistochemical evidence of enhanced calpain activity in nigral neurons in postmortem human tissues. *A*, Immunohistochemical detection of calpain-cleaved α-spectrin (38-4) viewed at low (20×) magnification revealed intense clusters of immunopositive staining in PD cases that were rarely observed in control cases. *B*, *C*, Confocal optical sectioning (0.6 μm) of calpain-cleaved α-spectrin immunoreactivity in pigmented dopamine neurons from a case without neurological condition (*B*) and from a case with Parkinson's disease (*C*) further revealed the cytoplasmic speckled appearance of 38-4-immunopositive staining that colocalized with neuromelanin (insets). *D*, Quantification of the proportion of pigmented neurons per section that displayed speckled calpain-cleaved α-spectrin immunoreactivity from PD and control tissues revealed a significant increase in calpain activity in subjects with PD (**52.83 ± 7.5 vs 9.17 ± 3.1%; Mann–Whitney rank-sum test; $p = 0.0079$). Positive immunostaining disappeared when the primary antibody was omitted (data not shown). Scale bars: *A*, 25 μm; *B*, *C*, 50 μm.

Nigral management of basal ganglia function

A second aspect of this study pertinent to the understanding of PD is that calpain inhibition conferred nigral neuroprotection without replenishment of striatal dopamine yet prevented deficits in locomotor behavior in MPTP-treated mice and normalized markers of BG activity. Historically, the symptoms of Parkinson's disease have been considered to be a consequence of diminished striatal dopamine. Indeed, dopamine replacement therapies have supported this concept. However, previous work has also suggested that the actions of L-Dopa may not be restricted to surviving dopamine terminals in the striatum, but the substantia nigra may too be a significant site for the actions of L-Dopa and dopaminergic regulation of movement (Robertson and Robertson, 1988, 1989; Crocker, 1997; Fox et al., 1998).

It has long been recognized that nigral dopaminergic neurons release dopamine not only from their axons projecting to the striatum but also from their dendrites (Bjorklund and Lindvall, 1975; Cheramy et al., 1981). Therefore dendro-dendritic release of dopamine by SNc neurons may be of particular importance in terms of the function of the BG in PD (Robertson and Robertson, 1989; Tseng et al., 1997; Fox et al., 1998; Gainetdinov et al., 1999; Collier et al., 2002). Results of this study present evidence to suggest that preservation of nigral integrity may provide preservation of dopaminergic-related motor function after loss of striatal nerve terminals. Although the precise nature of the functional benefit associated with nigral neuroprotection by calpain inhibition is presently not known, there are several possibilities pertaining to nigral dopaminergic regulation of BG function.

The first possibility involves the counterbalancing influences of serotoninergic inputs to the substantia nigra pars reticulata (SNr). There is increasing evidence that dopaminergic denervation after degeneration of nigrostriatal neurons results in increased serotoninergic innervation of the reticulostriatal pathway (Thibaut et al., 1995; Fox and Brotchie, 2000). Interestingly, this influence can be attenuated by direct administration of $5HT_{2C}$ receptor antagonists into the SN (Fox et al., 1998). The function of serotonin in this context is thought to exacerbate hypolocomotor activity. This notion is exemplified in DAT-deficient mice in which basal hyperactivity can be modulated by enhancing serotonergic activity, without affecting levels of striatal dopamine (Gainetdinov et al., 1999). In the present study, the prevention of SNc degeneration by calpain inhibition may have preserved the integrity of dopaminergic innervation of the SNr and therein acted to circumvent a serotonin-mediated hypolocomotion.

A second possibility relates to dopaminergic modulation of excitatory innervation of the SNr. Dopaminergic innervation of

the SNr is thought to modulate the activation states of glutamate receptors on excitatory synapses from the subthalamic nucleus (Wittmann et al., 2002). Thus, it has been proposed that SNr function, and hence BG output, may be succinctly modulated by nigral interactions of dopamine, glutamate, and GABA signaling (Kelly et al., 1987; Abarca et al., 1995; Matuszewich and Yamamoto, 1999; Wittmann et al., 2002). Under the circumstances of the present study, the preservation of the tonic innervation of the SNr by dendritic dopamine release may compensate for depleted striatal dopamine by modulating BG function.

Third, recent findings have also lead to the suggestion that extrastriatal dopamine may modulate striatonigral feedback pathways. Interestingly, intranigral infusion of glial cell line-derived neurotrophic factor in unilateral dopaminergic lesion rat models have been previously reported to reverse postsynaptic neuropeptide changes in the striatum, coincident with improved behaviors and without altering striatal dopamine concentrations (Lapchak et al., 1997; Tseng et al., 1997). These findings are also consistent with our present results. Taken together, this evidence suggests a role for extrastriatal modulation of BG-mediated motor behavior perhaps through nigral modulation of striatonigral projections (Collier et al., 2002).

Although calpain inhibition may be of benefit for the aspects of PD/MPTP-related behavior that were measured in this study, it is important to emphasize that other PD-related symptomology has not been examined and may not be improved by calpain inhibition alone. Interestingly, however, several previous studies have also reported significant protection of the SNc without preservation of striatal dopamine (Liberatore et al., 1999; Mandir et al., 1999; Vila et al., 2001). Because motor performance was not assayed in these studies, it is unclear whether protection of the SNc after MPTP by various strategies will prevent behavioral deficits or whether preservation of dopamine-related motor behaviors are selective to calpain inhibition. Nevertheless, these findings suggest that the complex circuitry of the basal ganglia likely maintains functionally redundant projections that can substitute for an absence of striatal dopamine, therein supporting our contention that the SNc may be a critical locus for dopamine-related functions in PD.

The present findings support a preeminent role for calpain-mediated proteolysis in neurodegeneration and impairment of dopaminergic functions in a model of Parkinson's disease. These findings provide evidence to support the central role of the SNc in PD and imply that motor dysfunction in PD may not be the singular result of a loss of striatal dopamine but instead may represent the manifestation of an imbalance among multiple integrated pathways of basal ganglia nuclei.

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Development/Plasticity/Repair

Enriched Environment Confers Resistance to 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine and Cocaine: Involvement of Dopamine Transporter and Trophic Factors

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We investigated, in mice, the influence of life experience on the vulnerability to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a major neurotoxin that induces a Parkinson's disease-like syndrome in humans, and to cocaine, a potent psychostimulant that promotes drug addiction. Our findings show that adult C57BL/6 mice raised in an enriched environment (EE) for only 2 months are significantly more resistant to both drugs compared with mice raised in a standard environment (SE). Indeed, EE mice showed decreased locomotor activity in response to cocaine (10 and 20 mg/kg) as well as a different pattern of *c-fos* expression in the striatum compared with SE mice. After MPTP treatment, SE mice showed a 75% loss of dopamine neurons, whereas EE mice showed only a 40% loss. The dopamine transporter plays a key role in mediating the effects of both drugs. We thus investigated the regulation of its expression. EE mice showed less dopamine transporter binding in the striatum and less dopamine transporter mRNA per dopamine neuron at the cellular level as demonstrated by *in situ* hybridization. In addition, enriched environment promoted an increase in the expression of brain-derived neurotrophic factor in the striatum. These data provide a direct demonstration of the beneficial consequences that a positive environment has in preventing neurodegeneration and in decreasing responsiveness to cocaine. Furthermore, they suggest that the probability of developing neurological disorders such as Parkinson's disease or vulnerability to psychostimulants may be related to life experience.

Key words: environmental enrichment; tyrosine hydroxylase; c-*fos*; dopamine transporter; BDNF; Parkinson's disease; drug of abuse

Introduction

Dopamine is a neurotransmitter that has been implicated in a wide variety of neurological and psychiatric disorders, including Parkinson's disease, attention-deficit hyperactivity disorder, schizophrenia, and drug abuse (for review, see Jaber et al., 1996). Dopamine neurotransmission is tightly controlled by its rapid reuptake through the dopamine transporter (DAT), a plasma membrane protein specific to dopamine neurons (Giros and Caron, 1993). We participated previously in the development of strains of mice lacking the DAT (Giros et al., 1996). Our results established not only the central importance of this transporter as the element key to maintaining a dopaminergic homeostasis (Jaber et al., 1999) but also its role in the behavioral and biochemical action of neurotoxins (Bezard et al., 1999; Fernagut et al., 2002) and of drugs of abuse (Spielewoy et al., 2000).

The etiology of brain diseases is often multifactorial and may be precipitated by inducers such as infective agents and neurotoxins. Unfortunately, the complex interactions between life experiences and the pathogenic effects of such a huge family of external events are currently unknown. For instance, life events such as stress, drug intake, or conditioned stimuli may have long-term repercussions on the whole organism and especially on the CNS (Nesse and Berridge, 1997; Nestler and Aghajanian, 1997). Several recent articles have provided striking evidence of the relevance of this issue to genetically induced brain diseases. Indeed, they show that life conditions can compensate for behavioral impairments provoked by two genetic mutations, one inducing motor disturbances mimicking Huntington's disease (Van Dellen et al., 2000) and the other a marked memory impairment (Rampon et al., 2000). An enriched environment combining both inanimate and social stimulation has also been shown to stimulate mammalian brain plasticity in dentate granule cell neurogenesis (Kempermann et al., 1997) and in glial cell proliferation (Walsh et al., 1969; Fiala et al., 1978).

Considering the well reported adaptive properties of mesencephalic dopaminergic neurons in various pathological situations (Zigmond and Stricker, 1985), we hypothesize that envi-

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ronmental conditions may prove beneficial in brain diseases affecting monoaminergic nuclei such as in drug addiction and Parkinson's disease. Here we describe the behavioral and molecular consequences of raising mice in an enriched environment (EE) and their differential response to a psychostimulant (cocaine) or to a pro-parkinsonian neurotoxin [1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)] compared with mice raised in a standard environment (SE). We show that EE decreases responsiveness to cocaine and protects against MPTP-induced neuronal loss. These effects would be mediated, at least in part, by a downregulation of the dopamine transporter, a mandatory target for psychostimulants (Jaber et al., 1996), and the obligatory neuronal gate to dopaminergic neurotoxins (Gainetdinov et al., 1997; Bezard et al., 1999). Furthermore, we suggest that this downregulation may be related, although not limited, to the upregulation of the brain-derived neurotrophic factor (BDNF).

Materials and Methods

Housing. Inbred C57BL/6 mice ($n = 144$) were used in this study and were housed in a temperature-controlled room under a 12 hr light/dark cycle with *ad libitum* access to food and water. Experiments were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) for the care of laboratory animals. The mice were raised together in groups of four after weaning (at 3 weeks of age) for 2 months either in an SE or in an EE. The SE consisted of common cage housing (30 × 15 × 15 cm). The EE constituted a larger (75 × 45 × 25 cm) cage constantly containing six to seven toys, which included a wheel and a small house that were randomly changed once per week (Rosenzweig and Bennett, 1996).

Cocaine treatments and locomotor activity measurement. After 2 months in either environmental condition, motor activity was measured in activity cages made of Plexiglas and aluminum wire mesh, as described previously (Deroche-Gamonet et al., 2003). Mouse locomotor responses to novelty were recorded during the first exposure to the activity cages. Mice were then acclimated to the activity cages for 2 consecutive days. On the fourth day, mice were first habituated to the activity cage for 50 min and then injected with the appropriate dose of saline vehicle (intraperitoneally) or cocaine (10 or 20 mg/kg) (Cooperative Pharmaceutique Française, Melun, France). They were then returned to the activity cages for a 50 min period during which motor activity was recorded. At the end of the 50 min recording period, mice were taken into another room and killed by decapitation; their brains were then processed for *in situ* hybridization.

MPTP treatments and measurement of N-methyl-4-phenylpyridinium ion contents. After 2 months in either environmental condition, animals were treated either with MPTP (20 mg/kg, i.p.) or with vehicle (0.9% NaCl solution) using a schedule designed to induce a large dopaminergic lesion (four injections at 2 hr intervals and a fifth injection 7 d later) as described previously (Gross et al., 2003; Meissner et al., 2003). All mice were killed 7 d after the last injection of MPTP (Jackson-Lewis et al., 1995). Brains were removed and processed for either immunohistochemistry, *in situ* hybridization, binding experiments, or *N*-methyl-4-phenylpyridinium ion (MPP^+) levels.

MPP^+ levels were determined as described previously (Przedborski et al., 1992). Both SE and EE mice were killed 2 hr after a single intraperitoneal injection of MPTP (20 mg/kg). Brains were removed rapidly, and the caudate–putamen complex was dissected out on ice. Tissue samples were sonicated in 10 vol of 5% trichloroacetic acid containing 20 μg/ml 4-phenylpyridine (Sigma, St. Louis, MO) as internal standard. Homogenates were centrifuged at 15,000 × *g* for 20 min at 4°C, and the contents of MPP^+ in the resulting supernatants were quantified by HPLC with UV detection. MPP^+ was monitored at 295 nm, and flow rate was set at 1.5 ml/min. Quantification was made by comparison of peak height ratios in the samples with those of the standards.

Stereology and counting of tyrosine hydroxylase-immunoreactive neurons. Free-floating mesencephalic serial sections were processed for TH immunohistochemistry and then counterstained with cresyl violet [Nissl staining (NS)] as described previously (Bezard et al., 1997). Cell counts were performed using a computer-based image analyzer (Visioscan v4.12; Biocom, Les Ulis, France). Unbiased stereological techniques were used to estimate cell numbers in the substantia nigra pars compacta (SNc) (Gundersen et al., 1988; West and Gundersen, 1990) as described previously (Gross et al., 2003; Meissner et al., 2003). Every fourth section of the SNc, the boundaries of which were chosen by examining the size and shape of the different TH-immunoreactive (TH-IR) neuronal groups and cellular relationships to axonal projections and nearby fiber bundles (German et al., 1996; Franklin and Paxinos, 1997), was counted. Volume was calculated using the following formula: $V_{(SNc)} = \Sigma S\ td$; where ΣS is the sum of surface areas, t is the average section thickness, and d is the distance between the sections (Theoret et al., 1999). Eight sections, the first being randomly chosen, were used, and optical dissectors were distributed using a systematic sampling scheme. Dissectors (50 μm length, 40 μm width) were separated from each other by 30 μm (x) and 20 μm (y). In these dissectors, the nuclei of the neurons in focus were counted (Gundersen et al., 1988). To be counted, a neuron had to be entirely inside the dissector or lying across the dissector border by more than half its surface (Gundersen et al., 1988). Only two consecutive borders on four sides of the dissector were considered (Gundersen et al., 1988). The following formula was used to estimate the number of NS and TH-IR neurons: $n = V_{(SNc)} (\Sigma Q^- / \Sigma V_{(dis)})$; where n is the estimation of cell number, V is the volume of the SNc, ΣQ^- is the number of cells counted in the dissectors, and $\Sigma V_{(dis)}$ is the total volume of the dissectors (Theoret et al., 1999). Mean estimated numbers of NS (structural marker) and TH-IR (phenotypical marker) neurons ± SEM were then calculated for each group of mice.

In situ *hybridization histochemistry.* The *in situ* hybridization procedure was performed as described previously (Jaber et al., 1995; Bezard et al., 2001a; Gross et al., 2003; Meissner et al., 2003) with oligonucleotide probes designed to recognize the following: D_1 dopamine receptor (D1R) (Dearry et al., 1990; Gross et al., 2003), D_2 dopamine receptor (D2R, mix of three oligonucleotides) (Dal Toso et al., 1989; Gross et al., 2003), preproenkephalin-A (PPE-A) (Tang et al., 1983; Gross et al., 2003), preproenkephalin-B (PPE-B) (Nawa et al., 1984; Gross et al., 2003), preprotachykinin (PPT) (Nawa et al., 1984; Gross et al., 2003), DAT (Jaber et al., 1999), BDNF (Wong et al., 1997), full-length tyrosine kinase neurotrophin receptor ($TrkB^{TK+}$) (Wong et al., 1997), the truncated form of the TrkB neurotrophin receptor ($TrkB^{TK-}$) (Wong et al., 1997), and c-*fos* (Deroche-Gamonet et al., 2003). Oligonucleotide probes were labeled by tailing using terminal deoxynucleotide transferase (Promega, Madison, WI) with ^{35}S-deoxy-ATP (NEN, Paris, France) at a specific activity of 2×10^9 cpm/μg. Sections were allowed to hybridize at 42°C for 18 hr in hybridization solution [50% formamide, 4× SSC, 10% dextran sulfate, 10 mM dithiothreitol, and labeled probe up to a final concentration of 3×10^6 cpm/ml]. After stringent washes, the slides were dehydrated and exposed to β-max Hyperfilm (Amersham Biosciences, Arlington Heights, IL) along with autoradiographic microscale standards (Amersham Biosciences) for 7 d. For anatomical visualization and microautoradiographic analyses, sections were dipped into LM-1 liquid emulsion (Amersham Biosciences), diluted to one-third concentration with water, exposed in the dark for 6 weeks, and then developed and counterstained with hematoxylin. Grain counting was done at 50× magnification, and neurons that showed a grain density greater than background were considered positive. The final data were converted into amount of radioactivity using a calibration curve constructed with the brain paste standards. Samples from individual animals were always analyzed in triplicate.

Dopamine transporter binding. To identify dopaminergic nerve endings, radiolabeling of [^{125}I](*E*)-*N*-(3-iodoprop-2-enyl)-2β-carbomethoxy-3β-(4′-methylphenyl) nortropane (PE2I) was performed from the stannyl precursor according to a method described previously (Guilloteau et al., 1998). After purification, [^{125}I]PE2I was obtained in a no-carrier-added form with a specific activity of 2000 Ci/mmol. Sections were incubated for 90 min at 25°C in pH 7.4 phosphate buffer [composed of the following (in mM): 10.14 NaH_2PO_4, 137 NaCl, 2.7 KCl, and 1.76 KH_2PO_4], containing 100 pM [^{125}I]PE2I as described previously (Bezard et al., 2001b,c). After washes and drying at room temperature, sections along

with calibrated [^{125}I] microscales (Amersham Biosciences) were exposed to β-radiation-sensitive film (Hyperfilm β-max; Amersham Biosciences) in x-ray cassettes for 3 d.

Analysis of autoradiographs. Densitomeric analyses of autoradiographs were performed using an image analysis system (Densirag V. D2.99; Biocom) as described previously (Bezard et al., 2001c; Gross et al., 2003; Meissner et al., 2003). The optical density was assessed in the rostral caudate–putamen (Franklin and Paxinos, 1997). Three sections per animal and per marker were analyzed by an examiner who was blind to the experimental condition. Optical densities were averaged for each animal and converted to amount of radioactivity bound compared with the standards. Mean ± SEM bound radioactivity was then calculated for each group.

Statistical analysis. Statistical analyses were performed using a two-way ANOVA. If significant, ANOVAs were followed by *post hoc t* tests corrected for multiple comparisons by the method of Bonferroni (Miller, 1981) or Newman–Keuls (for behavioral analyses). All data were normally distributed, and significance levels of *t* test comparisons were adjusted for inequality of variances when appropriate. Statistical analyses of both MPP^+ content and the number of silver grains per cell were performed using the Mann–Whitney test given the low numbers of animals per group. These analyses were completed using Intercooled Stata 6.0 (Stata Corporation, College Station, TX). A probability level of 5% ($p < 0.05$) was considered significant.

Results

Enriched mice are less responsive to novelty

When mice were first tested for locomotor reactivity, EE mice showed reduced locomotor activity in the new environment of measurement cages when compared with SE mice ($F_{(1,31)} = 13.1$; $p < 0.001$). Locomotor activity of mice raised in either environment decreased significantly with time ($F_{(11,341)} = 69.64$; $p < 0.0001$) (Fig. 1*a*), which indicated habituation. No time × environment interaction was observed, suggesting that the decrease in locomotor activity with time was similar in both groups ($F < 1$).

Mice were then habituated to the locomotor activity cages for 2 hr per day for 2 consecutive days. On the day mice were to receive a cocaine or saline injection, they were again placed in activity cages for 50 min and then given either solution. During this preinjection period, locomotor activity of mice raised in either environment was comparable ($F < 1$) (Fig. 1*b*).

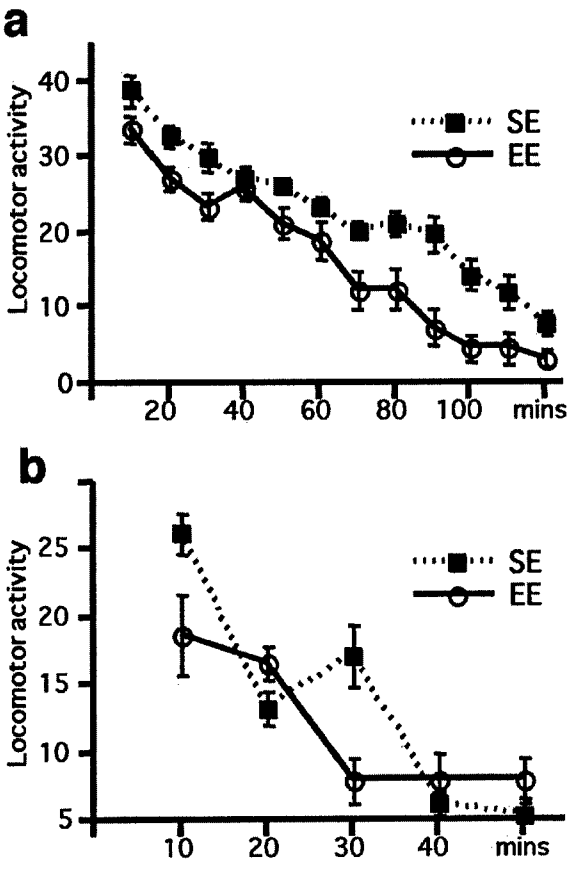

Figure 1. Mice raised in an enriched environment show decreased locomotor activity in response to novelty. Motor activity was measured in activity cages made of Plexiglas and aluminum wire mesh. Locomotor activity was measured every 10 min. *a*, On day 1, mice were put in measurement cages for 2 hr, and their locomotor response to novelty was measured during this first exposure. EE mice showed decreased locomotor activity compared with SE mice. *b*, Mice were acclimated to the activity cages for 2 consecutive days (days 2 and 3). The next day, they were again put in activity cages for 50 min before receiving cocaine. Note that the locomotor activity of SE and EE mice is not different before cocaine administration. Error bars are SEM.

Enriched mice are less responsive to cocaine

After habituation, mice received either saline or cocaine injections (intraperitoneally) at 10 or 20 mg/kg (Fig. 2*a*,*c*). Repeated-measures ANOVA, with time being the within-subject factor, showed that cocaine increased locomotor activity at 10 and 20 mg/kg ($F_{(3,12)} = 13.086$; $p < 0.001$ and $F_{(3,47)} = 57.5$; $p < 0.0001$, respectively). At both cocaine doses (10 and 20 mg/kg), locomotor activity decreased significantly with time ($F_{(4,48)} = 13.09$; $p < 0.0001$ and $F_{(4,188)} = 36.16$; $p < 0.0001$, respectively). In addition, we observed a time × environment interaction after cocaine at 10 and 20 mg/kg ($F_{(12,48)} = 5.73$; $p < 0.0001$ and $F_{(12,188)} = 11.86$; $p < 0.0001$, respectively), suggesting that the decrease in locomotor activity with time was different in relation to the environment. *Post hoc* analyses (Newman–Keuls) also showed that the increase in locomotor activity observed in EE mice was significantly less pronounced than that observed in SE mice at both 10 and 20 mg/kg cocaine doses ($p < 0.05$ for both doses) (Fig. 2*b*,*d*). Thus, EE mice were less responsive to cocaine (10 and 20 mg/kg) than SE mice (Fig. 2). There was no difference in mice treated with saline ($F < 1$) in relation to the environment.

Enriched mice have a different c-*fos* expression pattern after cocaine injection

Only a slight increase in c-*fos* expression was observed at 10 mg/kg cocaine, and this increase was below the detection limits of the quantification procedure, also as observed previously (Johansson et al., 1994). A single injection of cocaine (20 mg/kg) increased c-*fos* mRNA levels coding for c-*fos* in the striata of both EE and SE mice (Fig. 3) ($F_{(1,15)} = 8.08$; $p < 0.05$ and $F_{(1,12)} = 6.34$; $p < 0.05$, respectively). However, the pattern of c-*fos* expression was different according to the environment. Indeed, whereas c-*fos* mRNA expression was increased mainly in the medioventral part of the striatum of SE mice (Fig. 3*a*,*c*), it was mainly present in the dorsolateral part of the striatum in EE mice (Fig. 3*b*,*d*).

Enriched mice show decreased numbers of mesencephalic dopamine neurons

Under control conditions, EE animals had ~10% less dopaminergic neurons in the SNc than animals raised in SE (Fig. 4*a*). We also estimated the number of TH-IR neurons in the ventral tegmental area (VTA) to determine whether the lower numbers of TH-IR neurons in the SNc of EE mice were attributable to postnatal neuronal migration differences as proposed by Lieb et al. (1996). Both SE (8499 ± 338) and EE (8281 ± 210) saline-treated

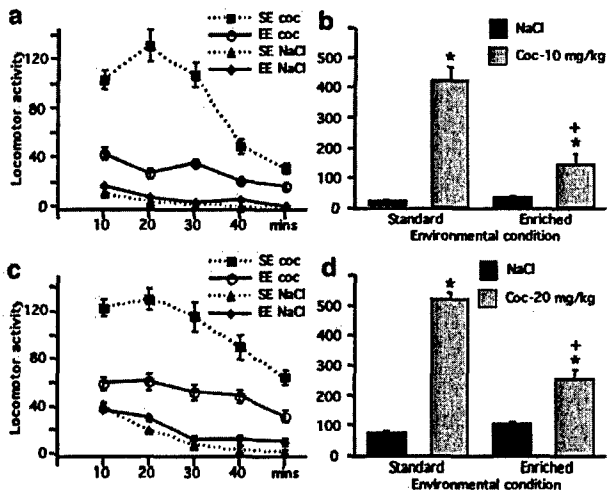

Figure 2. Mice raised in an enriched environment show decreased locomotor activity in response to cocaine (coc). Locomotor activity in SE or EE mice was measured after administration of 10 mg/kg cocaine (*a*, *b*), 20 mg/kg cocaine (*c*, *d*), or saline. Measurements were done every 10 min (*a*, *c*) and were also expressed with all time points pooled together (*c*, *d*). At both doses, cocaine induced a significant increase in the locomotor activity in both SE and EE mice. Note, however, that EE mice were less responsive to cocaine than SE mice (see Results for details). * indicates significantly different from saline-treated animals; + indicates significantly different from cocaine-treated mice raised in an SE.

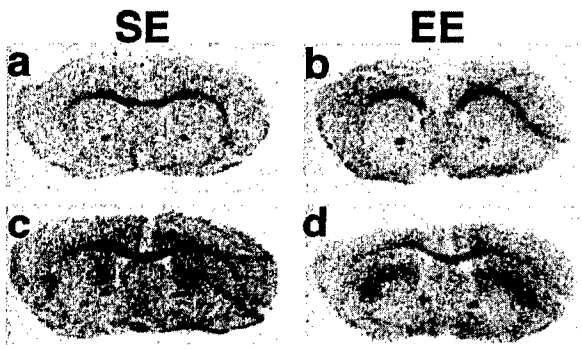

Figure 3. Cocaine-induced *c-fos* expression was altered in enriched mice. Saline treatment did not induce *c-fos* mRNA in our experimental protocol in either SE or EE mice after *in situ* hybridization. However, cocaine (20 mg/kg) significantly induced *c-fos* expression in both groups of mice, although in different striatal areas. Indeed, EE mice showed an expression restricted to dorsal areas that receive premotor and sensorimotor cortical projections, whereas SE mice showed expression in ventromedial areas, as found by most investigators working with mice housed in an SE and investigating *c-fos* mRNA (rather than protein). *a*, *b*, Saline-treated mice; *c*, *d*, cocaine-treated mice.

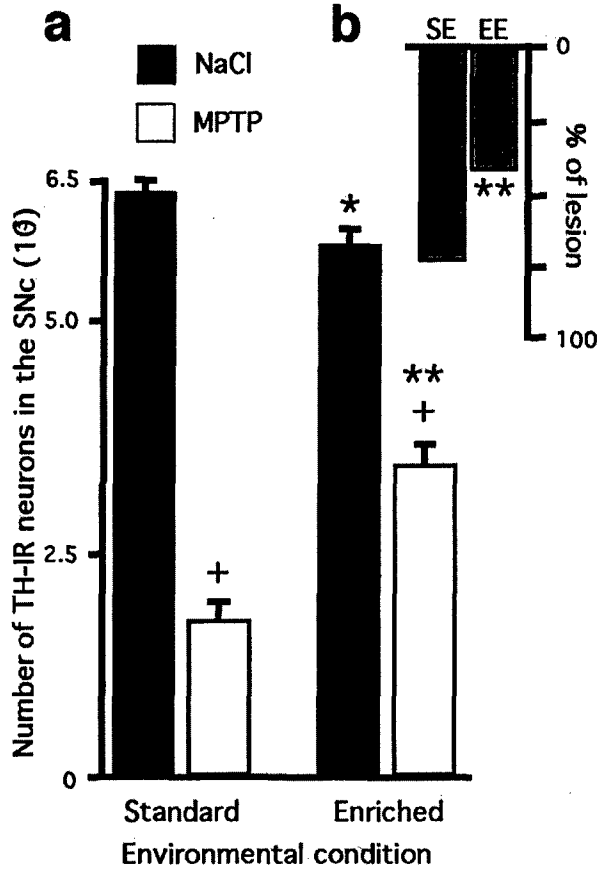

Figure 4. Living in an enriched environment provides protection against the pro-parkinsonian neurotoxin MPTP. *a*, Stereological counts of the number of TH-IR neurons in the SNc of saline- and MPTP-treated mice raised in SE or EE. Standard environment–NaCl, $n = 6$; SE–MPTP, $n = 7$; EE–NaCl, $n = 8$; EE–MPTP, $n = 6$. The enriched environment modified the number of TH-positive neurons ($F_{(1,26)} = 12.3$; $p < 0.01$) and interfered with the MPTP treatment ($F_{(1,26)} = 52.1$; $p < 0.0001$). Under control conditions, animals raised in an enriched environment had a lower number of TH-IR neurons than animals raised under standard conditions (*$p < 0.05$). After MPTP treatment, although both groups showed significant decreases in the number of TH-IR neurons compared with their respective controls ($^{+}p < 0.05$), the number of surviving neurons was twice as high in EE compared with SE animals (**$p < 0.05$). *b*, Thus, the MPTP-induced TH neuron loss was 73.4% in SE mice and 41% in EE mice. Errors bars are SEM.

mice showed similar estimated numbers of TH-IR neurons within the VTA ($F < 1$). This indicates that the 10% decrease in TH-IR neurons in the SNc of EE saline-treated mice could be the consequence of a higher neuronal elimination than a within-nuclei regulatory migration.

Enriched mice are more resistant to MPTP

A time interval of 7 d must be respected after MPTP exposure and before killing for the assessment of nigral degeneration by TH-IR neuronal count (phenotypical marker) in the mouse midbrain (Jackson-Lewis et al., 1995). However, because some cells can take up to 2 weeks to die, parallel counting of NS cells (structural marker) is required to control the validity of TH counting as a marker of cell death. After MPTP treatment, only 26.6% of TH-IR neurons of SE mice survived in accordance with previous studies using the same doses of MPTP in mice raised in standard housing conditions (Fig. 3*a*) (Gross et al., 2003; Meissner et al., 2003). Accordingly, NS cells drop from 6583 ± 144 in the saline–SE to 1747 ± 179 in the MPTP–SE.

In contrast, in EE animals, 58.5% of the TH-IR neurons survived (Fig. 4*a*), as confirmed by the drop in NS cells from 5962 ± 134 in saline–EE to 3499 ± 233 in MPTP–EE. Because the TH-IR and the NS counts matched, the cell death was completed. Thus, enriched environment increased the resistance of EE mice to MPTP by 199.7% (Fig. 4*b*).

MPP^{+} striatal content was not different ($p = 0.23$) in SE (6.9 ± 1.3 μg/mg tissue; $n = 3$) and EE (4.7 ± 0.5 μg/mg tissue; $n = 4$) between the two groups of mice that received a single injection of 20 mg/kg MPTP (Przedborski et al., 1992). Thus, the resistance to toxicity was not attributable to a lower delivery of MPTP to the brain after intraperitoneal injection, to reduced brain biotransformation of MPTP to MPP^{+}, or to diminished striatal mitochondrial monoamine oxidase B activity.

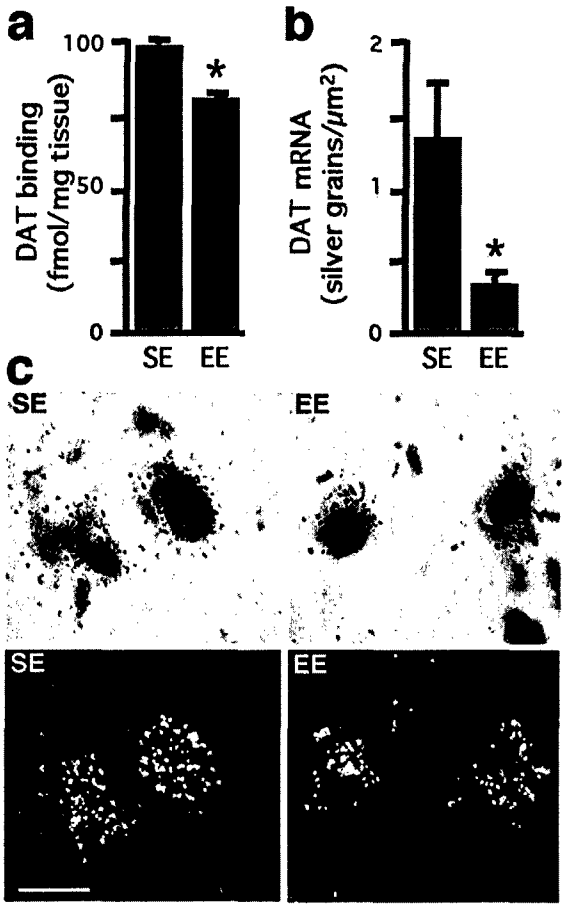

Figure 5. DAT binding is significantly decreased in the dorsal part of the striatum of EE mice (*$p < 0.05$; SE, $n = 8$; EE, $n = 8$). *a*, The 100% level in SE mice corresponds to a PE2I binding of 97.8 fmol/mg equivalent tissue. *b*, This decrease is most likely attributable to lower number of DAT per neuron, because *in situ* hybridization for DAT mRNA showed a 75% silver grain decrease in EE compared with SE mice on microautoradiography. *c*, Photomicrographs of neurons labeled by *in situ* hybridization using oligonucleotide probes complementary to DAT mRNA and viewed under direct (top) and polarized (bottom) light illumination. Scale bar, 15 μm. Error bars are SEM.

Decreased DAT expression may confer cocaine and MPTP resistance in EE mice

The effects of both cocaine and MPTP are known to be mediated through the DAT (Jaber et al., 1997; Bezard et al., 1999). To determine the role of DAT in this differential resistance to MPTP-induced toxicity and cocaine-induced locomotor responsiveness as well as the implication of this resistance, we performed binding experiments using [^{125}I]PE2I (Bezard et al., 2001c). Figure 5*a* shows that raising mice in an EE downregulates the striatal levels of DAT in the saline-treated animals, regardless of the zone defined within the striatum (i.e., dorsal and ventral). Indeed, ANOVA indicates a significant effect of enrichment ($F_{(1,31)} = 43.1$; $p < 0.0001$) but no effect of striatal zone ($F_{(1,31)} = 1.4$; $p = 0.25$) and no interaction between enrichment and striatal zone ($F < 1$).

This decrease in DAT levels is most likely attributable to a decrease in the number of DAT binding sites per remaining dopamine neuron. We reached this conclusion in light of data obtained from DAT mRNA *in situ* hybridization at the microautoradiographic level. Indeed, grain counting corresponding to specific labeling of DAT mRNA allows the evaluation of the intensity of *in situ* labeling per cell. As shown in Figure 5, *b* and *c*, we found that the grain density corresponding to DAT mRNA levels per cell was 75% lower in EE (0.32 ± 0.05) than in SE (1.32 ± 0.37) mice ($p = 0.01$). This suggests that mice raised in an enriched environment express much less DAT mRNA per dopamine neuron than mice raised in SE.

Growth factor expression increases after enrichment

BDNF is expressed in the mouse striatum (Wong et al., 1997; Guillin et al., 2001), the target area of substantia nigra dopamine neurons that were found to be decreased in EE mice. The substantia nigra is also a brain area in which DAT downregulation was observed in EE mice. For these reasons, because enrichment has been shown to induce expression of glial-derived neurotrophic factor and BDNF in the hippocampus (Young et al., 1999), and because simple motor enrichment also increases BDNF, we investigated the levels of mRNA expression of BDNF, TrkB^{TK+}, and TrkB^{TK-} neurotrophin receptors in the striatum.

TrkB^{TK+} (Fig. 6*a*,*b*) and TrkB^{TK-} (Fig. 6*c*,*d*) mRNA expression was not different in EE and SE mice ($F < 1$). BDNF, however, was increased in EE mice (e.g., 32.8 ± 1.4 fmol/mg equivalent tissue in dorsal striatum) compared with SE mice (e.g., 21.4 ± 1.2 fmol/mg equivalent tissue in dorsal striatum) (Fig. 6*e*,*f*). Indeed, ANOVA indicates a significant effect of enrichment ($F_{(1,31)} = 62.42$; $p < 0.0001$) but no effect of striatal zone ($F < 1$) and no interaction between enrichment and striatal zone ($F < 1$). Such an increase in BDNF expression suggests that the influence of the environment may be mediated through induction of growth factor expression.

No difference in other dopamine-regulated markers in relation to environment

Although the level of DAT was significantly affected by environmental conditions, the classical markers of dopamine transmission within the striatum were not. Indeed, D_1 (Fig. 6*g*,*h*) and D_2 (Fig. 6*i*,*j*) dopamine receptor mRNAs expression were not different between EE and SE mice ($F < 1$), regardless of the striatal area considered (e.g., the dorsolateral motor striatum or the nucleus accumbens). Expression of striatal PPE-A (Fig. 6*k*,*l*), PPE-B (Fig. 6*m*,*n*), and PPT (Fig. 6*o*,*p*) neuropeptide mRNA, known to be under the control of dopamine, was also not different between EE and SE mice ($F < 1$), further supporting the specificity of DAT regulation of expression within the nigrostriatal pathway.

Discussion

The present study shows that an enriched environment provides significant protection against both the locomotor and cellular effects of cocaine and MPTP-induced neurodegeneration. Such an effect probably involves an increase in expression of neurotrophic factors (e.g., BDNF as shown here) and a downregulation of the expression of DAT, the main target of psychostimulants and the neuronal gate to dopamine acting neurotoxins.

Responsiveness to cocaine is under environmental control

When raised in an enriched environment, C57BL/6 mice show reduced locomotor reactivity to a new environment compared with mice raised in an SE. Levels of activity in unfamiliar environments have been traditionally used as a measure of emotionality in rodents, although this is still a matter of debate (Day et al., 2001; Cabib et al., 2002). One obvious and very possible explanation of reduced activity in EE mice in a novel environment is that the measurement cages are much smaller and less attractive than their big home cages with renewed toys and social interaction.

From a molecular point of view, neurotoxins and psychoactive drugs produce their behavioral and cellular effects by acting

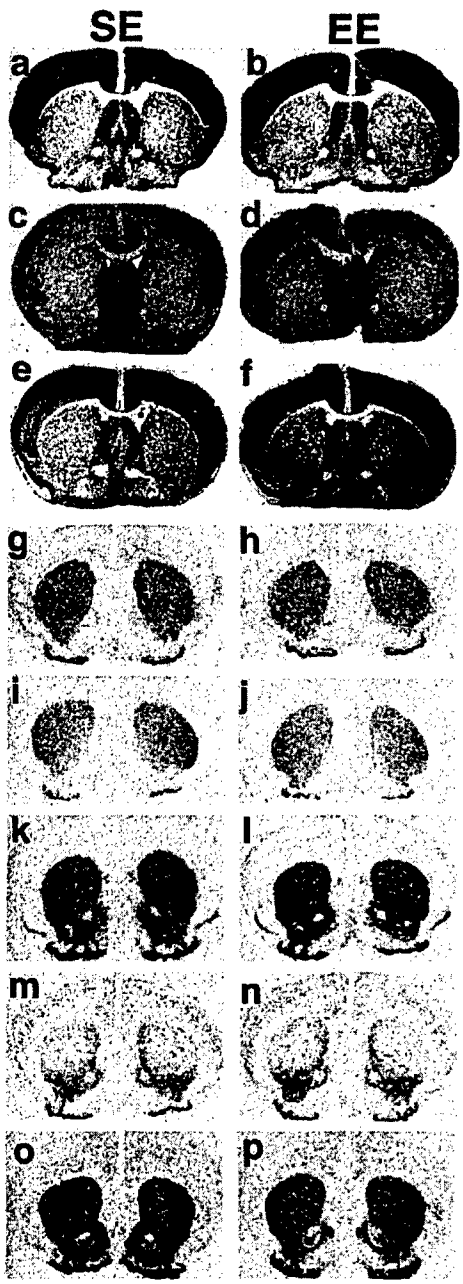

Figure 6. Enriched mice show increased expression of BDNF mRNA in the striatum as evidenced by *in situ* hybridization. Although the expression of both the full-length and the truncated form of the TrkB neurotrophin receptor [i.e., $TrkB^{TK+}$ (*a*, *b*) and $TrkB^{TK-}$ (*c*, *d*), respectively] is comparable in the SE and EE mice, BDNF mRNA expression (*e*, *f*) is significantly increased throughout the brain and especially in the striatum of the mice living in the enriched environment. The mRNA expression of several dopamine-sensitive markers [i.e., D1R (*g*, *h*), D2R (*i*, *j*), PPE-A (*k*, *l*), PPE-B (*m*, *n*), and PPT (*o*, *p*)] is not affected by the enriched environment.

at specific sites in the CNS, thus modifying neuronal targets (Nestler, 2001). This pharmacological explanation is confronted by several observations showing that drugs have different effects in relation to the circumstances related to their intake (Kelleher and Morse, 1968). Repetitive aggression of the organism may represent factors that predispose or precipitate drug self-administration, thus creating situations that overwhelm the capacity of self-adjustment (Piazza and Le Moal, 1998). Inspired by Pavlovian conditioned reflexes, Wikler (1948) was among the first to propose the role of associative factors in drug addiction. More recently, it has been demonstrated that a simple manipulation of the environment can modulate rats' reactions to an amphetamine injection. Indeed, rats showed increased locomotor activity when this drug is given in a novel environment (Badiani et al., 1995). In relation to the present study, environmental enrichment was shown previously to decrease intravenous amphetamine self-administration (Green et al., 2002). In accordance with these findings, we show that EE mice are less responsive to cocaine than SE mice. This finding suggests that initial personal experience may be crucial to determining resistance to drugs such as cocaine. Interestingly, EE mice show a different pattern of c-*fos* expression than SE mice after cocaine. c-*fos* mRNA was found mainly in ventromedial areas of the striatum of SE mice, as reported previously in studies using mice housed in an SE (Johansson et al., 1994). At variance, c-*fos* mRNA was expressed mostly in dorsolateral striatal regions in EE mice. Molecular changes in basal ganglia circuits are thought to play a crucial role in psychostimulant addiction and dependence (for review, see Nestler, 2001). An important determinant of striatal gene expression after psychostimulant treatments seems to be the excitatory input from the cortex (Cenci and Bjorklund, 1993). Dorsal striatal regions receive inputs mostly from sensory and motor cortical areas, suggesting that medial agranular (premotor) cortex and sensorimotor circuits are primarily involved in decreased cocaine response in EE mice.

Enriched environment and neuroprotection

Environmental enrichment can promote neuroplasticity and protect neurons, because evidence shows that these types of environments can promote increases in the complexity of dendritic arbors and synapses in the hippocampus and cerebellum (Black et al., 1989; Kleim et al., 1997) that can prevent age-related synaptic loss (Saito et al., 1994) and improve learning and memory (Kempermann et al., 1997; Nilsson et al., 1999; Young et al., 1999). Most if not all studies regarding the effects of enriched environment on neuronal injury and recovery after injury have focused on the cortical and hippocampal areas, long known to be responsive to environmental modifications. For instance, EE rats display less severe functional deficits caused by bilateral lesions of the frontal cortex in relation to reduced structural damage (Kolb and Gibb, 1991) and a better outcome after stroke (Johansson, 1996). Young et al. (1999) have also shown that enriched environment protects against excitotoxic injuries in a model of epilepsy. Nonetheless, this protection was chiefly the result of the prevention of kainate-induced seizures, because seizure duration is an essential determinant of the extent of neuronal injury in the hippocampus (Lothman and Bertram, 1993).

Although the overall impact of enriched environment on the brain circuitry of the dopamine system has not yet been investigated, the effects of motor activity on dopamine neuronal death have recently received attention. Indeed, forced limb use (i.e., a sort of motor enrichment) specifically renders dopamine neurons resistant to dopamine neurotoxins (Tillerson et al., 2001; Cohen et al., 2003), whereas forced nonuse (i.e., a motor impoverishment) does the opposite (Tillerson et al., 2002). In addition, physical activity has been shown to induce growth factor expression (Neeper et al., 1995; Gomez-Pinilla et al., 1997; Cohen et al., 2003), as reported here. Therefore, it is possible that the increased home-cage activity in the EE mice (in contrast to activity in a novel environment) may have been beneficial in that immediately after neurotoxin exposure, motor behavior can reduce neurotoxicity (Tillerson et al., 2001), whereas MPTP-related reduc-

tions in activity are likely to have exaggerated terminal loss (Tillerson et al., 2002).

Specific regulation of DAT expression

The magnitude of the protection that we report here is surprisingly elevated compared with pharmacological approaches aimed at reaching the same goal (for review, see Dawson and Dawson, 2002). Furthermore, we used a particularly demanding intoxication regimen to produce a severe lesion of the mesencephalic region (Gross et al., 2003; Meissner et al., 2003). To the best of our knowledge thus far, none of the hitherto proposed compounds have been tested using such a demanding paradigm that is supposed to overcome their neuroprotective capacity.

Because cocaine is a blocker of the DAT (Gonon et al., 2000) and MPTP is taken up by the same transporter (Gainetdinov et al., 1997) present exclusively on dopamine neurons (for review, see Giros and Caron, 1993), the DAT seemed to be a potential candidate for regulation by the environment on the basis of increased resistance to both drugs. Indeed, such a magnitude of neuroprotection could be achieved by blocking the DAT (Mayer et al., 1986) or by knocking out the DAT gene (Gainetdinov et al., 1997; Bezard et al., 1999). Using DAT knock-out mice, we demonstrated previously that the DAT is mandatory for MPTP-induced dopaminergic neurotoxicity (Gainetdinov et al., 1997; Bezard et al., 1999), and that it plays a crucial role in responsiveness to psychostimulants (Jaber et al., 1997; Gainetdinov et al., 1999).

The decrease in DAT binding that we observed may be attributable, at least in part, to the slight but significant 10% decrease in the number of dopamine neurons in the SNc (see below). However, such an explanation is ruled out by the fact that DAT mRNA expression was significantly reduced per nigral dopamine neuron, as we found by *in situ* hybridization at the microautoradiography level.

Many mechanisms are likely to mediate the effects of environment on both spontaneous and toxin-induced neuronal death. Inadequate trophic support from target-derived factors is known to play a role in physiological neuronal elimination. Exogenous administration or stimulus-induced expression of neurotrophic factors (e.g., through physical exercise) (Neeper et al., 1995) may increase the resilience of the brain to insults and neuronal damage (Gash et al., 1996; Bjorklund and Lindvall, 2000). As shown in the hippocampus of EE rats (Young et al., 1999) and in several brain structures of animals forced to exercise (Neeper et al., 1995; Gomez-Pinilla et al., 1997; Cohen et al., 2003), we report here an environmentally driven increase in BDNF expression in the striatum. Although such upregulated growth factor expression is unlikely limited to BDNF, the well known trophic factor activity of BDNF on dopamine neurons (Hyman et al., 1991) may play a role in DAT downregulation and in the subsequent resistance to MPTP and cocaine. A systematic investigation of EE on growth factor expression is needed to further characterize the respective role of these factors in this phenomenon.

SNc neurons are downregulated in EE mice

Perhaps one of the most intriguing findings is that mice raised in an EE, supposedly a positive environment that would prevent cell loss (Young et al., 1999), show 10% less TH-positive neurons in the SNc compared with mice raised in an SE. One possible explanation may rely on differential cell death through apoptosis, as has been shown in the hippocampus (Young et al., 1999). During postnatal development, dopaminergic neurons of the substantia nigra undergo natural cell death that fulfils the criteria for apoptosis (Jackson-Lewis et al., 2000). This process seems to play a critical role in regulating adult numbers of dopaminergic neurons. Indeed, normal embryonic and postnatal development of specific neuronal circuits that underlie various behaviors (sensory, motor, or cognitive) includes cell death (for review, see Honig and Rosenberg, 2000). During this process, viability of neurons depends on whether they are properly connected. The fine tuning of neuronal connections at vulnerable periods during development appears to be orchestrated by environmental cues, including the microenvironments in which individual neurons find themselves. These cues control critical developmental processes such as proliferation, migration, differentiation, synaptogenesis, and myelination (for review, see Rice and Barone, 2000). Spatial, temporal, or quantitative errors in the stimuli that initiate programmed cell death can result in pathological neural development. This is observed not only in typical neurodegenerative disorders such as Alzheimer's disease and Huntington's disease but also in several neurodevelopmental disorders such as schizophrenia and autism. Thus, the slight but significant decrease in the number of dopamine neurons of the SNc in EE mice may have beneficial consequences and may participate in resistance to insult by neurotoxins later on.

Conclusion

This study focuses on the long-term influence of environmental manipulation during adolescence, a critical period of development. The models that we used provided evidence of neuronal plasticity at the molecular level that may underlie the decreased responsiveness of EE mice to both cocaine and MPTP. Although direct extrapolation of these findings and comparable data in human conditions is to be taken with caution, the discovery of the molecular mechanism by which environmental factors can induce phenotypic effects seems an essential step for developing relevant models of brain diseases and, subsequently, efficient therapies.

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JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease

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Parkinson's disease (PD) is a neurodegenerative disorder characterized by loss of dopamine-containing neurons, but the molecular pathways underlying its pathogenesis remain uncertain. Here, we show that by eliminating c-Jun N-terminal kinases (JNKs) we can prevent neurodegeneration and improve motor function in an animal model of PD. First, we found that c-Jun is activated in dopaminergic neurons from PD patients and in the 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine (MPTP) mouse model of PD. Examination of various JNK-deficient mice shows that both JNK2 and JNK3, but not JNK1, are required for MPTP-induced c-Jun activation and dopaminergic cell demise. Furthermore, we have identified cyclooxygenase (COX) 2 as a molecular target of JNK activation and demonstrated that COX-2 is indispensable for MPTP-induced dopaminergic cell death. Our data revealed that JNK2- and JNK3-induced COX-2 may be a principle pathway responsible for neurodegeneration in PD.

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by selective and progressive demise of dopamine-containing neurons in the midbrain (1). Although mutated genes have been identified in some cases of inherited PD (2), genetic origin could not be found in most cases, which occur sporadically. Despite extensive investigations, the cause of PD remains unknown (3). Undoubtedly, the design of effective treatments for PD depends largely on our understanding of the molecular mechanisms leading to neurodegeneration, which is still incomplete. Yet, significant hints into PD pathogenesis have been yielded by the use of 1-methyl-4-phenyl-1,2,4,6-tetrahydropyridine (MPTP), a neurotoxin that replicates most of the neuropathological hallmarks of PD in humans, nonhuman primates, and other mammalian species, including mice (4). Although the MPTP mouse model departs from human PD in a few important ways, it offers a unique means to investigate, *in vivo*, molecular events underlying the demise of midbrain dopaminergic neurons (4).

Several lines of evidence indicate that excitotoxic mechanisms could play a role in dopaminergic neurodegeneration in PD (5), although the molecular pathway that leads to excitotoxic-mediated cell death remains elusive. We found previously that mice lacking the neuron-specific isoform of c-Jun N-terminal kinase (JNK), JNK3, display remarkable resistance to kainic acid-induced excitotoxicity (6). The JNK group of protein kinases phosphorylates the N-terminal activation domain of the transcription factor c-Jun, thereby regulating AP-1 complex transcriptional activity (7). Three genes (*Jnk1*, *Jnk2*, and *Jnk3*) with distinct expression patterns encode the JNK protein kinases (7). In contrast to JNK1 and JNK2, which are ubiquitously expressed, JNK3 is largely restricted to the brain (8). In the kainic acid-induced excitotoxicity model, the protective effect of JNK3 ablation in mutant animals is associated with marked reduction of c-Jun phosphorylation and decreased activity of the AP-1 transcription factor complex (6). A similar defect is observed in mice with a germ-line mutation of the c-*jun* gene that replaced the JNK phosphorylation sites (9). These data suggest that efficient transcription of JNK-dependent target genes is a necessary step in progression of neuronal cell death and prompted us to investigate JNK-mediated induction of deleterious gene(s) as a part of the cascade of events leading to neurodegeneration in parkinsonian syndromes.

Materials and Methods

Chemicals and Antibodies. MPTP·HCl (Sigma) was dissolved in 0.9% NaCl. The following antibodies were used: anti-phospho-c-Jun (Ser-73) (Cell Signaling Technology, Beverly, MA), c-Jun/AP-1 (N) and inhibitor of transcription factor NF-κB (IκBα; Santa Cruz Biotechnology), cyclooxygenase (COX) 2 (BD Biosciences, San Jose, CA), COX-1 (Cayman Chemical, Ann Arbor, MI), β-tubulin (Sigma), tyrosine hydroxylase (TH; Pel-Freez Biologicals), glial fibrillary acidic protein (GFAP; DAKO), and macrophage antigen complex 1 (MAC-1; Serotec). The NeuroTrace 500/525 green-fluorescent Nissl stain solution was purchased from Molecular Probes.

Animals and Treatment. Two- to 3-month-old male mice were used. JNK1-, JNK2-, and JNK3-deficient mice were generated as described (8). C57BL/6J, *COX-2*$^{-/-}$ (*Ptgs2*^{Tm1Jed}), and *p50*$^{-/-}$ (*Nfkb1*^{tm1Bal}) mice were purchased from The Jackson Laboratory. All strains were backcrossed at least six times to the C57BL/6 background. For studies using knockout mice, WT littermates were used as controls. Mice were injected i.p. four times (2-h intervals over 1 day) with either 20 mg/kg MPTP·HCl or a corresponding volume of saline alone. At the indicated time points, animals were killed and their brains were processed for further analysis. This protocol was in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and was approved by the Yale Animal Care and Use Committee.

Human Brain Postmortem Study. Autopsy brainstem tissue from control subjects and PD patients (n = 3 and 4, respectively), which were well characterized clinically and neuropathologically, were obtained from the brain bank at the Institut National de la Santé et de la Recherche Médicale U289. PD patients and control subjects did not differ significantly in their mean age at

Abbreviations: COX, cyclooxygenase; GFAP, glial fibrillary acidic protein; JNK, c-Jun N-terminal kinase; MAC-1, macrophage antigen complex 1; MPP+, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; SN, substantia nigra; SNpc, SN pars compacta; TH, tyrosine hydroxylase.

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death (controls, 78 ± 7 years; PD patients, 75 ± 5 years) or their mean interval from death to freezing of tissue (controls, 21.4 ± 5.6 h; PD patients, 18.8 ± 6.2 h). Brainstem tissue was fixed and processed for immunohistochemistry as described (10).

Western Blot Analysis. Ventral midbrain and striatum were quickly dissected and lysed in whole-cell lysis buffer (0.4 M NaCl/25 mM Tris·HCl, pH 7.5/1% SDS/50 mM NaF/1 mM Na_3VO_4), plus protease inhibitors (Roche Diagnostics). Protein lysates (30 μg) were resolved on SDS/10% PAGE gels, electrotransferred to Immobilon-P (Millipore) membranes, and probed with antibodies to phospho-c-Jun (1:1,000 dilution), c-Jun (1:1,000 dilution), β-tubulin (1:50,000 dilution), COX-2 (1:250 dilution), COX-1 (1 μg/ml), or IκBα (1:250 dilution), followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. Blots were visualized by chemiluminescence (SuperSignal; Pierce).

Immunohistochemistry. At the indicated time points, mice were fixed by transcardial perfusion of 4% paraformaldehyde/50 mM NaF/2 mM Na_3VO_4. Free-floating sections encompassing the entire midbrain and striatum were prepared by using a cryostat. For TH, GFAP, and MAC-1 immunostaining, tissue sections were incubated with primary antibodies overnight at 4°C. Biotin-conjugated secondary antibody incubation, avidin–biotin-conjugated peroxidase, and 3,3′-diaminobenzidine reactions were performed as described (11). Nissl counterstaining was performed by using standard procedures. Total numbers of TH- and Nissl-positive cells were counted in one hemi-brainstem by using stereological methods. For double immunofluorescent staining, sections were first incubated overnight with primary antibodies against phospho-c-Jun, followed by Cy3-conjugated secondary antibodies, and then with either NeuroTrace fluorescent Nissl stain for 20 min or primary antibodies against TH, GFAP, and MAC-1.

Measurement of Striatal Monoamine Levels. Mice were killed 7 days after MPTP or saline injections, and their striata were quickly dissected. Striatal tissue was then processed for HPLC measurement of dopamine and homovanillic acid content by means of electrochemical detection as described (11).

Measurement of Striatal 1-Methyl-4-phenylpyridinium Ion (MPP^+) Levels. Mice were killed at 90 and 180 min after the last MPTP injection, and their striata were recovered and processed for HPLC using UV detection (295-nm wavelengh) to measure MPP^+, as described elsewhere (11).

Rotarod Trial. The Rotarod apparatus (Ugo Basile, Trieste, Italy) consists of five rotating drums (3 cm in diameter) separated by flanges that enable five mice to be recorded simultaneously. Mice (at least 10 animals per group) were first pretrained three times (1 h apart) at 6–7 days after MPTP or saline injections by using an accelerating mode. After this conditioning period, the time on the rod (with a maximum recording time of 240 sec) was recorded for successive rotational speeds, and the overall rod performance of each animal was calculated by the trapezoidal method (12).

Gene Expression Analysis. WT and *Jnk3*$^{-/-}$ mice (three animals per group) received a single injection of kainic acid (30 mg/kg), and their hippocampi were dissected by 6 h after intoxication. Hippocampal tissue was then placed into TRIzol reagent (GIBCO/BRL) and homogenized, and total RNA was prepared according to the manufacturer's instructions. Probe synthesis and labeling, hybridization, and scanning were performed as reported (13). In this study, we used two different microarrays (Mu11K subA and subB) representing >10,000 mouse genes and expressed sequence tags. Results were analyzed by using GENECHIP software (Version 3.1, Affymetrix, Santa Clara, CA).

COX-2 RT-PCR. Total RNA (2 μg), isolated from hippocampus by using TRIzol reagent, was reverse-transcribed by using SuperScript reverse transcriptase (GIBCO/BRL) in a total volume of 25 μl. From this reaction, 2 μl was used as a template for PCR amplification with *Tsg* DNA polymerase (Lamda Biotech, St. Louis). The COX-2 cDNA was amplified by using the following primers: 5′-AAAACCGTGGGGAATGTATGAGC-3′ and 5′-GATGGGTGAAGTGCTGGGCAAAG-3′. The PCR program consisted of 25 cycles at 94°C for 20 sec, 60°C for 40 sec, and 72°C for 40 sec.

Results and Discussion

c-Jun Is Activated in Dopaminergic Neurons in both PD Brains and MPTP-Intoxicated Mice. JNK mediates activation of c-Jun by means of the phosphorylation of two serine residues (Ser-63 and Ser-73) located in the N-terminal domain. To test whether MPTP-induced dopaminergic cell death is associated with JNK-induced c-Jun activation, we first evaluated expression of phosphorylated c-Jun by Western blot analysis using an antibody raised against c-Jun phosphorylated at Ser-73. MPTP intoxication causes a significant increase of phosphorylated c-Jun in the ventral midbrain, which can be observed as early as 5 h after the first intoxication (Fig. 1*A*). It remains elevated for 1 day and returns to its basal level at 7 days. In the striatum, an increased level of phosphorylated c-Jun was observed also, albeit it appeared earlier (3 h postinjection) and was less sustained (data not shown). These data indicate that JNK-mediated c-Jun activation in dopaminergic nerve terminals precedes that in substantia nigra pars compacta (SNpc) cell bodies. Relevant to this interpretation is the finding that MPP^+ (the toxic metabolite of MPTP) accumulates first in striatal dopaminergic terminals rather than in dopaminergic soma (14). To get further insights into the cellular localization of phosphorylated c-Jun, we examined its distribution by double immunofluorescent staining performed on mesencephalic brain sections. In saline-injected animals, no phospho-c-Jun-specific labeling could be detected in nigral neurons identified by Nissl staining (Fig. 1 *B*, *G*, and *L*). In contrast, by 12 h after MPTP treatment, there was a strong phospho-c-Jun immunoreactivity in some nigral neurons (Fig. 1 *C*, *H*, and *M*). These neurons were dopaminergic, as demonstrated by double labeling with anti-TH antibodies (Fig. 1 *D*, *I*, and *N*). Double staining using antibodies raised against glial cell markers (GFAP and MAC-1 for astrocytes and microglial cells, respectively) showed that c-Jun was phosphorylated exclusively in dopaminergic neurons (Fig. 1 *E*, *J*, and *O* for GFAP and *F*, *K*, and *P* for MAC-1). Interestingly, there was also a dynamic change in the subcellular distribution of phosphorylated c-Jun. By 8 h, phospho-c-Jun labeling was observed exclusively in the cytosol (Fig. 1*H Inset*), whereas it was nuclear by 12 h (Fig. 1 *M Inset* and *N*). These data indicate that phosphorylation of cytosolic c-Jun in dopaminergic neurons triggers its translocation from the cytoplasm to the nucleus possibly by means of the stabilization of the protein (15). Hence, phospho-c-Jun accumulation in the nucleus is consistent with its role in AP-1-dependent transcriptional activity.

To investigate whether dopaminergic cell death in PD might be associated with similar subcellular redistribution of c-Jun, we next surveyed the expression pattern of c-Jun/AP-1 transcription factor in the mesencephalon of postmortem human brain from patients with idiopathic PD and matched control subjects. Midbrain sections were subjected to immunohistochemistry using a polyclonal antibody raised against the N-terminal domain of c-Jun. At the cellular level, c-Jun/Ap-1 staining (Fig. 2*A*, arrows) was observed throughout the mesencephalon in dopaminergic neurons identified by their neuromelanin content (Fig. 2*A*, arrowheads). In control subjects, c-Jun/AP-1 immunoreactivity was detected exclusively in the perikarya and processes of dopaminergic neurons (Fig. 2*A*), whereas nuclear staining could

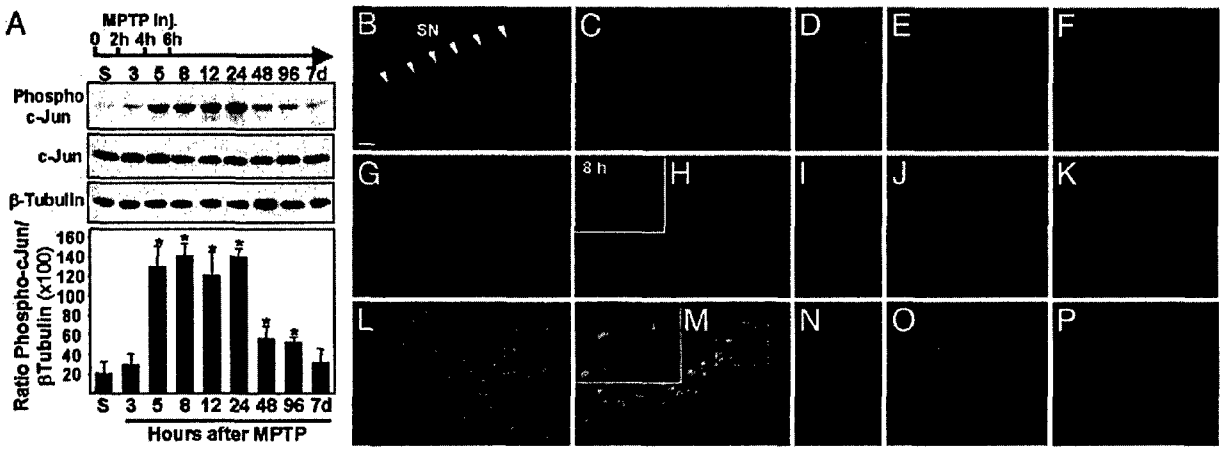

Fig. 1. c-Jun is activated in dopaminergic neurons after MPTP intoxication. (*A*) Western blot analysis for phospho-c-Jun in the mouse mesencephalon after MPTP injections (arrow). Compared with saline-injected mice (S), the phospho-c-Jun expression level increases in a time-dependent manner after MPTP intoxication. Data represent mean ± SEM. (n = 3–5). *, $P < 0.01$, compared with saline-injected mice (two-tailed t test). (*B–P*) Double immunofluorescent staining for phospho-c-Jun (*G–K*), Nissl (*B* and *C*), TH (*D*), GFAP (*E*), or MAC-1 (*F*) on ventral midbrain tissue sections. The expression of phospho-c-Jun in the SN (arrowheads in *B*) was virtually absent in saline-injected mice (*G*) but increased greatly by 8 h after MPTP treatment (*H Inset*). At 12 h, phospho-c-Jun expression (*H*) was still elevated in neuronal cells (*M*) and translocated to the nucleus (*M Inset* is a higher magnification of the area in the dotted rectangle). Neurons displaying phospho-c-Jun immunoreactivity were positive for the dopaminergic cell-specific marker TH (N). Phospho-c-Jun staining never colocalized with the astrocytic marker GFAP (*O*) or the microglial cell marker MAC-1 (*P*). [Scale bar represents 60 μm (*B*, *C*, *G*, *H*, *L*, and *M*), 10 μm (*D*, *I*, and *N*), and 20 μm (*H* and *M Insets*).]

be observed occasionally in pigmented dopaminergic neurons from PD brains (Fig. 2 *B* and *C*, arrows). Specificity of labeling was confirmed by absorption of the antiserum with the control peptide. In these conditions, no staining was observed (data not shown). Because the phosphorylation of c-Jun induces its translocation into the nucleus, our postmortem data suggest that c-Jun/AP-1 is activated in some dopaminergic neurons in the pathology. Quantitative analysis of c-Jun-positive pigmented neurons in the SNpc demonstrated that the proportion of melanized neurons with nuclear c-Jun staining was increased significantly (Mann–Whitney U test, $P < 0.005$) in the PD patient group compared with control subjects (mean proportion in percentage ± SEM: 1.5 ± 0.08 and 0.02 ± 0.01 for PD patients and control subjects, respectively), suggesting that c-Jun activation may be related to PD physiopathology.

JNK-Deficient Mice Are More Resistant to MPTP. Data from postmortem material and the MPTP mouse model suggest that JNK-mediated c-Jun activation might be implicated in PD-associated neurodegeneration. To test this hypothesis, we compared MPTP toxicity in mutant mice deficient in JNK1, JNK2, or JNK3 ($Jnk^{-/-}$) with that of their WT littermates. In saline-injected animals, there were no differences in dopaminergic cell content between WT and individual JNK knockout mice (Fig. 3 *A* and *B*, histological data not shown for $Jnk1^{-/-}$). This finding suggests that ablation of a single JNK isoform has no impact on the normal development of the nigrostriatal pathway, a result consistent with some of our investigations on other brain regions

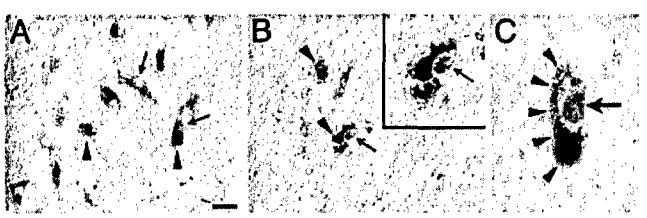

Fig. 2. Immunohistochemical detection of c-Jun/AP-1 in transverse sections of control (*A*) and parkinsonian (*B* and *C*) SNpc. c-Jun immunoreactivity (arrows) was observed in perikarya and processes of dopaminergic neurons, identified by their neuromelamin content (arrowheads). (*B* and *C*) Note examples of melanized dopaminergic neuron displaying strong immunoreactivity in the nucleus (arrow), which can be distinguished clearly from neuromelanin pigments (arrowheads). [Scale bar represents 40 μm (*A* and *B*), 15 μm (*B Inset*), and 10 μm (C).]

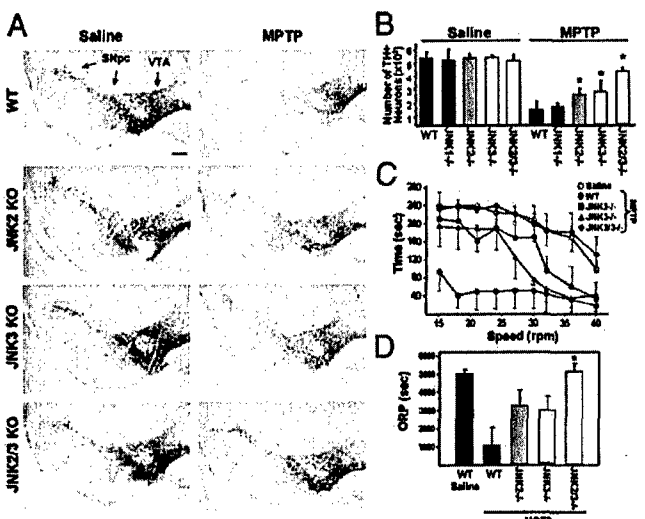

Fig. 3. Comparison of MPTP-induced nigrostriatal pathway injury in WT and $Jnk^{-/-}$ mice. (*A*) Peroxidase immunohistochemistry for TH on midbrain sections from saline- and MPTP-injected WT and JNK-deficient mice. (Scale bar represents 200 μm.) (*B*) Stereological counts of TH-positive cells in the SNpc at 7 days after MPTP intoxication. JNK2 or JNK3 ablation elicits sparing of TH-positive neurons after MPTP treatment, as compared with WT mice. Dual deletion of JNK2 and JNK3 increases the protective effect further. *, $P < 0.01$, compared with MPTP-injected WT mice (Mann–Whitney U test). (*C* and *D*) Motor performance of saline- or MPTP-treated WT and JNK-deficient mice measured on a Rotarod. The mean times on the rod recorded for increasing rod-rotation speeds (8–15 mice per group; *C*) and the mean overall rod performance (ORP, see *Materials and Methods*) for each group of mice (*D*) show that MPTP-intoxicated JNK-deficient animals display significant improvement of motor functions compared with MPTP-treated WT mice. *, $P < 0.05$, by Mann–Whitney U test.

(6, 8). However, whereas MPTP induced an 80% loss of TH-positive nigral cells in WT animals, the number of dopaminergic neurons was reduced by only 50% in both *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$ intoxicated animals (Fig. 3*B*). Nissl staining and counting of neurons indicate that TH-positive cell loss after MPTP treatment is due not to MPTP-induced down-regulation of the TH marker but rather to dopaminergic cell destruction (see Table 1, which is published as supporting information on the PNAS web site). In contrast, despite the fact that JNK1 is expressed in the mesencephalon (8), MPTP-treated *Jnk1*$^{-/-}$ mice displayed as much dopaminergic cell death as their WT littermates (Fig. 3*B*). These observations indicate that JNK2 and JNK3 are the main JNK isoforms implicated in stress-induced dopaminergic cell death in the MPTP mouse model.

The partial effect of *Jnk2* or *Jnk3* ablation opens the question whether these two JNK isoforms could compensate for each other. To investigate this issue, we generated compound mutants of JNK2/JNK3 knockouts. Unlike compound mutants JNK1/JNK2 (8), the JNK2/JNK3-null mice develop normally with no apparent nigrostriatal pathway abnormalities. Yet, the protective effect afforded by the dual deletion of the *Jnk2* and *Jnk3* genes was more pronounced than that found in mice carrying a deletion of either one of these JNK isoforms (only 15% of TH-positive cells were lost in *Jnk2/Jnk3*$^{-/-}$ mice; Fig. 3 *A* and *B*). It appears, therefore, that both JNK2 and JNK3 are required for MPTP-induced dopaminergic cell death *in vivo*. In addition to the sparing of nigral dopaminergic cell bodies, we observed that striatal dopaminergic nerve terminals in JNK-deficient mice were also more preserved than in their WT counterparts. Indeed, whereas MPTP induced a 94% reduction in striatal dopamine content in WT animals, it reached only 55% in double JNK2/JNK3 null mice (see Table 2, which is published as supporting information on the PNAS web site). In JNK2- or JNK3-deficient mice, the levels of dopamine were also markedly attenuated after MPTP treatment, albeit less than in WT animals but more than in JNK2/JNK3 compound mutants (90% and 80% reduction in *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$, respectively). Consistent with the dopaminergic cell-count results, MPTP-treated JNK1-deficient mice showed striatal dopamine depletion similar to that of WT animals, and therefore, there is no protective effect of JNK1 ablation in this model of PD (data not shown).

The symptomatic manifestations in PD (motor dysfunction) are due to a profound reduction in striatal dopamine content caused by the loss of dopaminergic nerve fibers in the striatum (1). Because JNK deficiency is associated with a protective effect on the nigrostriatal pathway after MPTP intoxication, we sought to determine whether JNK mutant animals have attenuated motor deficits. The locomotory ability of mice was evaluated 6–7 days after saline or MPTP treatment by using a Rotarod (12). We found that MPTP induces a profound reduction of motor performance in WT animals even at low speeds (15–25 rpm; Fig. 3*C*). This defect could be reversed when the animals were given L-dopa (levodopa) and benserazide (50 and 2.5 mg/kg, respectively, twice a day for 3 days), suggesting that the observed motor dysfunction is a direct consequence of nigrostriatal pathway injury (data not shown). Yet, MPTP-treated *Jnk2*$^{-/-}$ and *Jnk3*$^{-/-}$ mice show a much better ability to perform the test but only at low speeds, as evidenced by the fact that their performance dramatically dropped at rod rotations >30 rpm (Fig. 3*C*). Consequently, the overall rod performance score was not statistically different between MPTP-treated WT and JNK2 or JNK3 knockout animals (Fig. 3*D*). In sharp contrast, MPTP-intoxicated double JNK mutants perform as well as saline-injected mice regardless of the speed of the rod (Fig. 3 *C* and *D*). Thus, these results show that ablation of JNK2 and JNK3 not only protects dopaminergic neurons against MPTP-induced neurodegeneration but also improves the motor function in this animal model of PD.

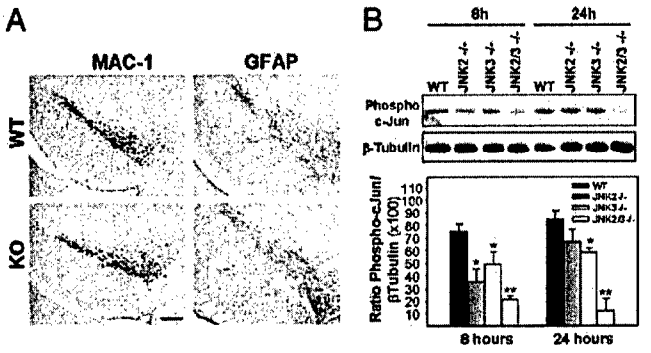

Fig. 4. Comparison of glial reaction and c-Jun activation in MPTP-intoxicated WT and JNK-knockout (KO) mice. (*A*) Immunohistochemical analysis for MAC-1 and GFAP indicates that MPTP-induced glial reaction is not altered in JNK-null mice as compared with WT animals. (Scale bar represents 300 μm.) (*B*) Western blot analysis for phospho-c-Jun expression in the mesencephalon after MPTP treatment shows that JNK2 and JNK3 are required for MPTP-induced c-Jun phosphorylation. Data represent mean ± SEM for three or four mice per group. *, $P < 0.05$; **, $P < 0.01$, compared with MPTP-treated WT mice (two-tailed *t* test).

MPTP Metabolism, Microglial Cell Activation, and c-Jun Phosphorylation in JNK-Deficient Mice. One of the first rate-limiting factors in MPTP toxicity is the conversion of MPTP into MPP^+ by means of the monoamine oxidase B enzymatic activity (3). To confirm that resistance of JNK-deficient mice was due to ablation of *Jnk* genes and not to alteration of MPTP metabolism, we measured striatal levels of MPP^+ after systemic injection of the toxin. JNK-deficient and WT mice exhibit similar striatal MPP^+ content (see Table 3, which is published as supporting information on the PNAS web site). Thus, poor drug delivery or metabolism was not likely to account for the lower susceptibility of *Jnk*$^{-/-}$ mice to MPTP toxicity. Microglial cell activation associated with neurodegenerative processes represents another important component of MPTP toxicity (11, 16). These cells can exert deleterious effects by means of multiple pathways, including production of nitric oxide (17). It has been reported that the microglial response to MPTP arises as early as 12 h postinjection, a time point that is significantly earlier than the active phase of dopaminergic neuron degeneration (18). Moreover, recent evidence has suggested that the JNK pathway is crucial for microglial cell activation (19). It is, therefore, conceivable that targeted deletion of *Jnk* may protect dopaminergic neurons from MPTP toxicity indirectly by means of the inhibition of microglial cell activation. To test this hypothesis, we surveyed midbrain expression of MAC-1. *Jnk*$^{-/-}$ and WT mice exhibited a comparable level of activated microglial cells distributed throughout the SN (Fig. 4*A*). This finding suggests not only that *Jnk*$^{-/-}$ and WT mice were subjected to an equal noxious stress but also that JNK ablation does not interfere with microglial cell activation. Similar results were obtained regarding astrocytosis (Fig. 4*A*). These findings are consistent also with the observation that MPTP-induced JNK/c-Jun activation is confined strictly to neurons (Fig. 1). Thus, it is unlikely that JNK deficiency confers protection by means of the inhibition of glial cell-associated deleterious mechanisms. Yet, early (8 h) and late (24 h) MPTP-induced c-Jun phosphorylation in the mesencephalon was decreased markedly in JNK2- and JNK3-deficient mice and inhibited completely in compound JNK2/JNK3 mutants (Fig. 4*B*). Thus, these data demonstrate that disruption of the *Jnk2* and *Jnk3* genes suppressed MPTP-induced phosphorylation of c-Jun and presumably AP-1 transcriptional activity in nigral dopaminergic neurons *in vivo*.

***COX-2* Is a Target Gene of JNK Activation.** It has been suggested that, at least in certain conditions, JNK-induced neuronal cell death is mediated by sustained levels of AP-1 transcriptional activity

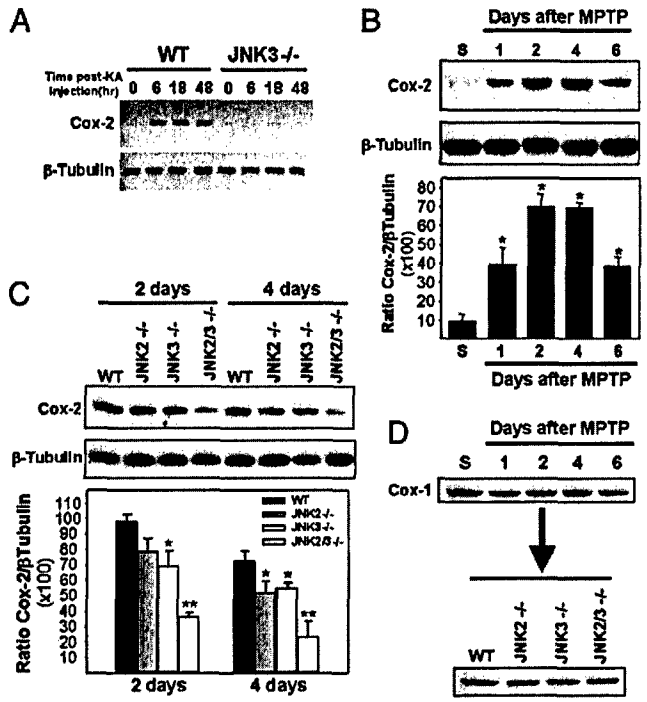

Fig. 5. JNK signaling pathway is required for COX-2 induction after neuronal noxious stress. (*A*) Expression of Cox-2 mRNA in the hippocampus of kainic acid-treated mice, as assayed by RT-PCR, is abolished in *Jnk3*-null mice. (*B*) Western blot analysis for COX-2 expression in the mesencephalon after MPTP treatment in WT mice for the indicated times. *, $P < 0.01$, compared with saline-injected mice. (*C*) MPTP-induced COX-2 expression in the mesencephalon is attenuated in JNK-deficient mice. *, $P < 0.05$; **, $P < 0.01$, compared with MPTP-treated WT mice (two-tailed *t* test). In *B* and *C*, data represent mean ± SEM for three or four mice per group. (*D*) Unlike COX-2 expression, COX-1 expression is not altered after MPTP treatment.

rather than by JNK effects on nontranscriptional targets. This contention is supported by the finding that kainic acid-induced neuronal cell death *in vivo* required functional c-Jun phosphorylation sites (9). Efficient transcription of target genes seems, therefore, to be required for JNK-induced neuronal cell death. To identify such genes and evaluate whether they might be implicated in JNK-mediated neurodegeneration *in vivo*, we took advantage of the kainic acid-induced excitotoxicity model that we used previously to study and characterize the phenotype of the *Jnk3*$^{-/-}$ mice (6). We compared gene expression profiles between kainic acid-treated WT and *Jnk3*$^{-/-}$ mice by using GeneChip microarrays. We found ≈50 genes to be differentially expressed with higher expression levels in WT mice than in JNK3-deficient animals (fold increase ranging from 2.2 to 22; data not shown). Importantly, expression of the *Jnk3* gene was found to be missing in *Jnk3*$^{-/-}$ mice but was present in WT animals, thus attesting that the experiment had been conducted successfully. Focusing on genes that have been implicated previously in neuronal cell death processes, we found that expression of *Cox-2* was up-regulated in WT but not in *Jnk3*$^{-/-}$ mice. To confirm this finding, we monitored hippocampal *Cox-2* mRNA expression by RT-PCR. As expected, kainic acid-treated WT mice exhibited a strong induction of *Cox-2* mRNA, whereas *Jnk3*$^{-/-}$ showed virtually no induction (Fig. 5*A*), suggesting that JNK3 is essential for *Cox-2* induction during excitotoxicity.

MPTP-Induced COX-2 Expression Requires Functional JNK but Not NF-κB Pathway. COX, also known as prostaglandin H synthase, is a rate-limiting enzyme for prostanoid synthesis that is present in two main isoforms: COX-1 and COX-2. COX-1 is expressed constitutively in many cell types, whereas COX-2 expression is generally induced by cytokines and other stress-induced stimuli (20). In brain, COX-2 is present in selected neurons and its expression is up-regulated in numerous pathological conditions, including Alzheimer's disease (21). Relevant to the present study are the findings that *Cox-2*$^{-/-}$ mice are strongly resistant to excitotoxicity-induced neuronal cell death (22) and that COX-2 expression is up-regulated in PD brains (23). These data suggest that JNK-mediated COX-2 induction might represent an important step in the cascade of molecular events leading to neurodegeneration in parkinsonian syndromes. To test this hypothesis we first examined whether MPTP induces COX-2 expression in mouse brain. Western blot analysis revealed that COX-2 is barely expressed in the mesencephalon of saline-injected animals but strongly induced after MPTP challenge (Fig. 5*B*). In contrast, change in COX-2 expression was at no time detected in the striatum (data not shown). In the mesencephalon, induction of COX-2 expression became noticeable by 1 day, peaked at 2 to 4 days, and persisted for at least 6 days. These data indicate that JNK/c-Jun activation after MPTP injection precedes COX-2 induction by at least 20 h, a result consistent with the hypothesis that COX-2 expression depends on JNK-induced AP-1 transcriptional activity. If this hypothesis is true, COX-2 up-regulation should be attenuated in MPTP-injected JNK-deficient mice. We tested this possibility by comparing the expression level of COX-2 in WT and *Jnk*$^{-/-}$ mice killed at 2 or 4 days after MPTP treatment. COX-2 expression was moderately attenuated in single JNK knockout mice and almost abolished in compound JNK mutants (Fig. 5*C*). These data further support the assertion that MPTP-induced COX-2 expression requires a JNK2/JNK3-mediated signaling pathway. Furthermore, the recent finding that MPTP-induced COX-2 expression is confined strictly to dopaminergic neurons is in agreement with the present results (23). Interestingly, JNK-induced COX expression was specific to the COX-2 isoform because brain COX-1 expression was not changed after MPTP treatment and was not altered in JNK-deficient mice, as compared with WT mice (Fig. 5*D*).

The regulation of COX-2 expression by the JNK signaling pathway described here is consistent with several reports describing the presence of putative AP-1 sites in the *Cox-2* gene promoter region (24, 25). However, other transcriptional regulatory elements also have been characterized and shown to be essential for the transcriptional induction of *Cox-2* in different cell types and experimental conditions. Among these other transcriptional regulatory elements, NF-κB sites are thought to play a central role (20, 26). To investigate whether the NF-κB signaling pathway might be involved in MPTP-induced COX-2 expression, we first analyzed activation of this signaling pathway in WT mice after MPTP treatment. The degradation of IκBα, taken as an index of NF-κB activation, was analyzed by using the time points used previously to study the phosphorylation of c-Jun (Fig. 1*A*). IκBα degradation was seen by 12 h after MPTP treatment, became maximal by 2 days, and was not observed further by 7 days (see Fig. 6*A*, which is published as supporting information on the PNAS web site). Thus, activation of the JNK/c-Jun signaling pathway slightly precedes that of NF-κB. Importantly, IκBα degradation in MPTP-treated JNK-deficient mice was similar to that in WT mice, suggesting that JNK ablation does not interfere with the NF-κB signaling pathway. We next tested whether MPTP-induced COX-2 expression was altered in mice with a defective NF-κB signaling pathway. The NF-κB transcription factor is constituted, for the most part, of the p50 (NF-κB1) and p65 (RelA) polypeptides. Because disruption of the mouse RelA locus leads to embryonic lethality, we used *p50*$^{-/-}$ mice that develop normally but display functional defects in immune responses (27). In contrast to JNK-deficient

mice (Fig. 5C), COX-2 expression in $p50^{-/-}$ mice after MPTP was similar to that of WT mice (Fig. 6B). Importantly, analysis of histological and neurochemical parameters in MPTP-lesioned $p50^{-/-}$ mice showed that the magnitude of the nigrostriatal pathway injury is identical to that of WT mice (data not shown). Thus, these results indicate that COX-2 induction occurs independently of the NF-κB transcriptional pathway and that this transcription factor has no significant role in the MPTP-induced neurodegenerative process.

COX-2 Is Instrumental in MPTP-Induced Neurodegeneration. To evaluate the impact of COX-2 up-regulation on dopaminergic cell death, we then compared the neurotoxic effect of MPTP in $Cox\text{-}2^{-/-}$ mice and their WT littermates. TH-positive cell counts in the SN revealed that $Cox\text{-}2^{-/-}$ mice were more resistant (2.6-fold increase in TH-positive neuron survival) to MPTP-induced dopaminergic cell death than WT mice (see Fig. 7, which is published as supporting information on the PNAS web site), suggesting the importance of this JNK-dependent target molecule in neuronal cell death.

Conclusion

Our study provides genetic evidence for the regulation of COX-2 by the JNK signaling pathway in mammals *in vivo*. Moreover, we have shown here that JNK-mediated COX-2 transcriptional induction is essential for MPTP-induced dopaminergic cell death. Although JNK-mediated COX-2 induction appears to be necessary for MPTP-induced neurodegeneration, it remains uncertain whether it is sufficient. Indeed, we cannot rule out the possibility that other JNK deleterious molecular targets may participate in this mechanism also. Yet, we believe that this possibility might not be the case because pharmacological inhibition of JNK activation in MPTP-treated $Cox\text{-}2^{-/-}$ mice does not mitigate the neurodegenerative processes further (23). Importantly, our results are consistent with previous studies. However, one major advantage to our gene targeting approach, as compared with pharmacological inhibition (28) or gene transfer of the JIP1-derived JNK binding domain inhibitor (29), is the ability to investigate the role of a single JNK isoform independently of inhibition of its counterparts. This aspect is of major importance; mounting evidence indicates that different JNK isoforms have distinct biological functions and might not all be implicated in stress-induced neuronal cell death (7). Thus, our study demonstrates also that not all JNK isoforms contribute equally to stress-induced dopaminergic cell death. Indeed, although JNK2 and JNK3 mediate MPTP-associated stress response in dopaminergic neurons, JNK1 does not. Yet, this finding is consistent with recent *in vitro* investigations using cultured cerebellar neurons in which both JNK2 and JNK3, but not JNK1, were found to be activated selectively by stress and responsible for c-Jun activation (30). Thus, our data further support the idea that various JNK isoforms are likely to exert different functions. For instance, acute activation of JNKs after noninvasive environmental stimuli (such as physical restraint) does not result in neurodegeneration, suggesting that the JNK signaling pathway may play important physiological roles in normal neuronal function (31). These data underline, therefore, the importance of developing specific JNK inhibitors for therapeutic applications because long-term pan-JNK inhibition in the nervous system might not be desirable. Finally, postmortem examination of parkinsonian brain tissue suggests that the deleterious signaling pathway described here may be activated and may induce dopaminergic cell death in the human disease; evidence of c-Jun activation and up-regulated expression of COX-2 in dopaminergic neurons was found in PD patients (23). Altogether, our data indicate that JNK2/JNK3 and/or COX-2 represent promising molecular targets for the development of future therapeutic intervention in PD.

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Pathogenesis of nigral cell death in Parkinson's disease☆

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Abstract

Parkinson's disease (PD) is primarily a sporadic condition which results mainly from the death of dopaminergic neurons in the substantia nigra. Its etiology remains enigmatic while its pathogenesis begins to be understood as a multifactorial cascade of deleterious factors. As of yet, most insights into PD pathogenesis are derived from toxic models of PD and show that the earlier cellular perturbations arising in dopaminergic neurons include oxidative stress and energy crisis. These alterations, rather than killing neurons, trigger subsequent death-related molecular pathways including elements of apoptosis. The fate of dopaminergic neurons in PD may also be influenced by additional factors such as excitotoxicity, emanating from the increased glutamatergic input from the subthalamic nucleus to the substantia nigra, and the glial response that arises in the striatum and the substantia nigra. In rare instances, PD can be familial, and those genetic forms have also provided clues to the pathogenesis of nigrostriatal dopaminergic neuron death including abnormalities in the mechanisms of protein folding and degradation as well as mitochondrial function. Although more remains to be elucidated about the pathogenic cascade in PD, the compilation of all of the aforementioned alterations starts to shed light on why and how nigral dopaminergic neurons may degenerate in this prominent disease, that is PD.

Keywords: Parkinson's disease; MPTP; Neurodegeneration; Pathogenesis

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after the Alzheimer's dementia. It is estimated that more than one million individuals in the United States of America alone are affected with this disabling disease and that more than 50,000 new cases arise each year [1]. PD is progressive with a mean age at onset of 55, and with an incidence that increases markedly with age [2]. Clinically, PD is characterized by the cardinal features of tremor at rest, slowness of voluntary movements, rigidity, and postural instability [1]. Like many other neurodegenerative diseases, PD presents itself mainly as a sporadic condition, meaning in absence of any genetic linkage, but in rare instances, PD can also arise as a simple Mendelian trait, linked to defects in a variety of genes [3]. Although, clinically and pathologically, sporadic and familial PD may differ on several significant aspects, they all share the same biochemical brain abnormality, namely a dramatic depletion in brain dopamine [2].

The reason why PD patients exhibit low levels of brain dopamine stems from the degeneration of the nigrostriatal dopaminergic pathway, which is made of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compact and whose projecting axons and nerve terminals are found in the striatum [2]. Yet, it is important to emphasize the fact that the neuropathology of PD is far from being restricted to the nigrostriatal pathway, and histological abnormalities can be found in many other dopaminergic and even non-dopaminergic cell groups [2]. The second most prominent neuropathological feature of PD is the presence of intraneuronal inclusions called Lewy bodies (LBs) in the few remaining nigral dopaminergic neurons [2]. LBs are spherical eosinophilic cytoplasmic aggregates composed of a variety of proteins, such as α-synuclein, parkin, ubiquitin and neurofilaments, and they can be found in every affected brain region [2].

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Over the past few decades a large core of data originating from clinical studies, autopsy materials, and in vitro and in vivo experimental models of PD has been accumulated, which led us to begin to have some level of understanding of the pathogenesis of sporadic PD [2]. Available data would argue that the mechanism of neuronal death in PD starts with an otherwise healthy dopaminergic neuron being hit by an etiological factor, such as mutant α-synuclein. Subsequent to this initial event, it is proposed that a cascade of deleterious factors is set in motion within that neuron made not of one, but rather of multiple factors such as free radicals, mitochondrial dysfunction, excitotoxicity, neuroinflammation, and apoptosis to cite only some of the most salient. Still based on this proposed scenario, all of these noxious factors will interact with each other to ultimately provoke the demise of the injured neuron.

Despite unquestionable major advances made in the molecular and cellular biology of PD and other neurodegenerative diseases which brought us closer than ever to being capable of unraveling the pathogenesis of PD, several critical questions remain unanswered. In this paper, three pending questions pertinent to the mechanisms of neuronal death in PD are discussed and form the body of this review. To be discussed first will be the question of what do we know about the nature of the pivotal factors and the sequence in which they act within the proposed pathogenic cascade that leads to neuronal death in PD. Second is the question to know whether the overall neurodegenerative process in PD is truly a cell autonomous process will be briefly addressed. Finally, one cannot avoid discussing the contribution of rare, inherited forms of PD to our current understanding of the pathogenesis of sporadic of PD.

2. Nature and sequence of action of pathogenic factors in PD

The current model of pathogenesis that most investigators in the field utilize has been outlined above. To confirm the actual role of these different presumed factors and the sequence by which they, respectively, intervene in this multifactorial cascade has been primarily, if not exclusively, studied in toxic experimental models of PD, which are numerous. Findings from these models and especially from that produced by the parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) indicate that the initial cellular perturbations include inhibition of mitochondrial respiration. Indeed, soon after the systemic administration of MPTP to mice, its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP^+), does concentrate in the mitochondrial matrix, where it binds to complex I of the electron transport chain [4]. MPP^+ binding interrupts the flow of electrons along the mitochondrial electron transport chain, thereby leading to an increased production of reactive oxygen species (ROS), especially of superoxide radicals [4]. Previous work has clearly demonstrated that the magnitude of MPP^+-related ROS production within the mitochondria is a dose-dependent phenomenon [5]. The pathogenic significance of such a local excess of ROS production is supported by the demonstration that mitochondrial aconitase activity is reduced in ventral midbrain of MPTP-treated mice [6]. MPP^+-related loss of electron flow is also associated with a drop in ATP production [4], which in vivo is found only in susceptible areas of the brain such as ventral midbrain and striatum [7]. Remarkably, this work shows that ATP deficit develops very rapidly after MPTP injection and lasts only for a few hours, as by one day post-MPTP tissue content in ATP seem to return to normal values [7].

In addition to provoking mitochondrial oxidative stress and energy crisis, MPP^+ also interacts with synaptic vesicles through its binding to vesicular monoamine transporter-2 [8]. In so doing, MPP^+ translocates into synaptic vesicles where it stimulates the extrusion of synaptic dopamine [9,10], reminiscent of the effect of methamphetamine. The resulting excess of cytosolic dopamine can readily undergo autooxidation, thus generating a huge burst of ROS, subjecting nigral neurons to an oxidative stress [11]. Alternatively, oxidation of cytosolic dopamine can also be catalyzed by enzymes such as cyclooxygenase-2 [12], which is upregulated in the remaining nigral dopaminergic neurons in both MPTP-treated mice and in human post-mortem samples [13]. Supporting this proposed event is our demonstration that cyclooxygenase-2 promotes dopamine–quinone formation following MPTP injection, and the production of protein-bound 5-cysteinyl-dopamine adducts in the brain of MPTP-injected mice [13]. In addition, excess of cytosolic dopamine can stimulate the formation of neuromelanin [14], a dark intraneuronal pigment implicated in the greater susceptibility of nigral neurons to PD neurodegeneration [15]. Although it remains uncertain how neuromelanin does actually contribute to the demise of dopaminergic neurons, it has been hypothesized that this pigment can do so by a *macromolecule crowding effect* or by playing the role of *intraneuronal toxic reservoir* by binding different transitional metals, such as iron, and various toxicants, such as MPP^+ [16].

All of the studies discussed above point toward the superoxide radical being pivotal in MPTP neurotoxicity. However, superoxide radical is known to not be highly reactive, and thus it is unlikely that it may be directly responsible for the damage inflicted by MPTP. Instead, it is much more likely that superoxide neurotoxicity results from superoxide reacting with other reactive molecules to generate what are called *secondary reactive species* of much greater tissue damaging potential, such as peroxynitrite. Consistent with this view are the demonstrations that the production of peroxynitrite, evidenced by quantifying tissue content of protein-bound 3-nitrotyrosine, is increased after MPTP injection [17] and that peroxynitrite is likely implicated in the nitrative post-translational modifications

of pathogenically meaningful proteins such as α-synuclein and parkin [18,19].

Collectively, the aforementioned findings indicate that early pathogenic events following MPTP administration include mitochondrial and cytosolic oxidative stress and ATP deficit. Yet, when one compares the time course of these cellular perturbations with the actual phase of neuronal degeneration found after MPTP injections, it clearly appears that oxidative stress and energy crisis precede the peak of dopaminergic neuronal death in the substantia nigra of mice which is situated around 24–48 h after the last injection of MPTP [20]. This finding suggests that these early events may kill some dopaminergic neurons, but that most of the neurons injured by this parkinsonian toxin fail to succumb to this early attack. Instead, it is believed that rather than killing a large number of neurons, early oxidative stress and energy crisis activate cell death-related molecular pathways which are the real executioner of the injured neurons. Among these are c-Jun *N*-kinases [21], cyclin-dependent kinases [22], and various components of the apoptotic machinery [23]. To illustrate the critical role of these molecular pathways in the MPTP-induced neurodegenerative process, two studies that pertain to apoptosis will be discussed here. First is the work done on the pro-cell death protein Bax, demonstrating that not only is Bax highly expressed in nigral dopaminergic neurons, but that ablation of Bax renders mice more resistant to the dopaminergic neurotoxicity of MPTP [24]. The second study deals with apoptotic protease activating factor 1 (APAF-1), one of the critical components of the apoptosome complex [25]. In the latter work, the authors have unilaterally delivered a viral vector expressing a dominant negative mutant of APAF-1 by stereotaxic injection in the substantia nigra [25]. Then, they have subjected these mice to a systemic administration of MPTP and found that the blockade of APAF-1 did mitigate the death of dopaminergic neurons [25]. These two studies clearly demonstrate the importance of downstream molecular pathways such as apoptosis in the death of nigral dopaminergic neurons and are consistent with the sequential pathogenic model proposed above.

3. Is the neurodegenerative process in PD cell autonomous?

This question is of critical importance both for pathogenic and therapeutic reasons. Indeed, it is quite important to determine whether the demise of nigral dopaminergic neurons strictly results from the cellular perturbations that arise within these neurons due to the disease's etiology, or from a complex interaction between what are called intrinsic and extrinsic perturbations. The answer to this question is complicated and far from straightforward. For instance, if one reviews the body of literature on cultured neurons exposed to MPP^+ or overexpressing PD-causing proteins such as α-synuclein [2], there is no doubt that these catecholaminergic neurons die in absence of any other intervening exogenous factors such as other cell types. However, when one looks at more complex systems, such as post-mortem tissues from PD patients or in vivo experimental models of PD, there is mounting evidence that indicates that the surroundings of the nigral dopaminergic neurons appear to play a critical role in influencing the fate of these dopaminergic cells. Among the potential culprits is the increased glutamatergic input to the nigra and which originates from the hyperactive subthalamic nucleus [26,27]. Moreover, the glial response that is found in both striatum and nigra of PD patients and MPTP-mice is also likely to exert deleterious effects on the remaining dopaminergic neurons [28]. This view has led many investigators, including those in my laboratory, to aggressively examine the potential role of neuroinflammation in the pathogenesis of PD. This important topic, however, will not be reviewed here as it is discussed in-depth in the accompanied paper written by Dr E. Hirsch. Based on these data and those presented by Dr Hirsch in this special issue, it is our opinion that several factors, exterior to dopaminergic neurons, contribute to creating a hostile environment, which increases the stress on already compromised dopaminergic neurons present in the vicinity. These factors, while likely not capable of initiating the disease, are nevertheless likely to amplify the neurodegenerative process and stimulate the progression of a chronic disease such as PD. If this view is correct, one then must take into account those exogenous factors if one wishes to completely and accurately comprehend the pathogenic cascade underlying the neurodegenerative process of PD and to develop effective neuroprotective therapies for this illness.

4. Insights from the rare inherited forms of PD

Until recently, all of the hypotheses regarding the cause and the mechanisms of PD neurodegeneration came from investigations performed in autopsy material from sporadic PD cases or in neurotoxic models [2]. However, less than a decade ago this situation changed with the identification of a mutation in α-synuclein associated with PD in an Italian kindred [29]. Since then, four additional PD-causing genes have been identified, and a linkage has been reported for at least five more. Although rare, these inherited cases have opened new directions of research which have already led to the integration within the proposed pathogenic cascade of new molecular components. In particular, familial cases of PD have brought to our attention the potential importance of protein aggregation and abnormalities in protein turnover in the overall process provoking neurodegeneration in PD. The contribution to our understanding of the pathogenesis of sporadic PD from the different PD-causing mutations has been discussed elsewhere [3], and thus readers

interested in this question are urged to consult this paper. Since the publication of this latter review, several important new findings have been published and, of these, three are worth discussing briefly.

As mentioned above, protein degradation has emerged as a potentially important theme in PD pathogenesis, especially in the context of alterations of the proteasome/ubiquitin pathway. Yet, protein degradation does not solely rely on the proteasome/ubiquitin pathway, but also on autophagy. With respect to autophagy, it has been reported that both wild-type and mutant α-synuclein can be degraded by lysosomal enzymes and that both bind to the autophagy chaperone [30]. Remarkably, however, mutant α-synuclein binds with much greater avidity to the autophagy chaperone than its wild-type counterpart [30]. Furthermore, contrary to wild-type α-synuclein which after binding to the chaperone is rapidly taken up by autophagic vacuoles for lysosomal degradation, mutant α-synuclein remains tightly attached to the chaperone and is never taken up or degraded [30]. These striking observations indicate that mutant α-synuclein fails to be properly degraded by the chaperone-mediated autophagy (CMA). It can, thus, be speculated that part of the neurotoxic mechanism of mutant α-synuclein may be related to the blockade of CMA and the consequent accumulation of unwanted proteins that are no longer eliminated by CMA.

The two other studies to be discussed here pertain to observations made on DJ1 and PINK1, the products of genes which upon mutation are linked to familial forms of PD, whose sub-cellular locations are mitochondrial [31,32]. In both cases, it was shown that either the abrogation of the mitochondrial localization or the loss of activity of these proteins renders cells subjected to mitochondrial poisons or proteasome inhibitor more prompt to degeneration [31,32]. Although the exact functions of DJ1 and PINK1 remain to be elucidated, these results already indicate that wild-type DJ1 and PINK1 assume some type of mitochondrial functions which confer resistance of the whole cell to a variety of stressors. Worth noting is the fact that, at this point there is no evidence to indicate that the putative mitochondrial action of DJ1 or PINK1 is linked to the electron transport chain machinery.

5. Conclusion

In this short review, an attempt has been made to stress the fact that the current consensus regarding the pathogenesis of sporadic PD is based primarily on information gathered from neurotoxic models of the disease. Based on these data, it appears that nigral dopaminergic neuron degeneration does not result from the action of a single deleterious factor, but rather from the convergence of multiple pathogenic factors. Many of these noxious factors emanate from within the dopaminergic neurons, whereas several others originate from outside the dopaminergic neurons such as glutamatergic input and glial cells.

We also know by now that while uncommon, there is much to learn from these rare familial cases of PD linked to gene mutations. In a matter of a few years, thanks to several elegant investigations performed in genetic cases of PD, we have become aware of the importance of excess protein aggregation with respect to mechanisms of neuronal death, perturbations in protein degradation systems such as proteasome and autophagy, and accumulation of unwanted proteins. These genetic cases have also shed light on new mitochondrial mechanisms other than those related to the electron transport chain which may have great pathogenic significance.

Despite enormous advances, it is fair to conclude by saying that much remains to be done to completely unravel the pathogenesis of PD. Among specific aspects that may deserve particular attention are the identification of where within the proposed pathogenic cascade do mutation-related deleterious mechanisms intersect with those mediated by the parkinsonian toxins. Why nigral neurons are more vulnerable than other dopaminergic neurons to the PD neurodegenerative process is also paramount to a comprehensive understanding of the neurobiology of this prominent neurodegenerative disease.

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APPENDIX 15

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MPTP and SNpc DA Neuronal Vulnerability: Role of Dopamine, Superoxide and Nitric Oxide in Neurotoxicity. Minireview.

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Parkinson disease (PD) is a common neurodegenerative disease of unknown origin that is characterized, mainly, by a significant reduction in the number of dopamine neurons in the substantia nigra pars compacta (SNpc) of the brain and a dramatic reduction in dopamine levels in the corpus striatum. For reasons that we do not know, the dopamine neuron seems to be more vulnerable to damage than any other neuron in the brain. Although hypotheses of damage to the dopamine neuron include oxidative stress, growth factor decline, excitotoxicity, inflammation in the SNpc and protein aggregation, oxidative stress in the nigrostriatal dopaminergic system garners a significant amount of attention. In the oxidative stress hypothesis of PD, superoxide, nitric oxide and dopamine all conspire to create an environment that can be detrimental to the dopamine neuron. MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), the tool of choice for investigations into the mechanisms involved in the death of dopamine neurons in PD, has been used extensively in attempts to sort out what happens in and around the dopamine neuron. Herein, we review the roles of dopamine, superoxide and nitric oxide in the demise of the dopamine neuron in the MPTP model of PD as it relates to the death of the dopamine neuron noted in PD.

Keywords: MPTP; Substantia nigra pars compacta; Neurotoxicity; Dopamine; Superoxide; Nitric oxide; Oxidative stress; Parkinson's disease

INTRODUCTION

Most neurodegenerative diseases involve specific subsets of neurons. In the case of Parkinson's disease (PD), a common neurodegenerative disorder characterized behaviorally by resting tremor, rigidity, akinesia/bradykinesia and postural instability, these are mainly, though not exclusively, the dopaminergic neurons in the substantia nigra pars compacta (SNpc) whose fibres project to the corpus striatum. There are, at present, 1 million PD patients in the United States alone, with 50,000 newly diagnosed cases each year (Fahn and Przedborski, 2000). These cases include both familial and sporadic PD, of which sporadic PD appears to be the more common (Dauer and Przedborski, 2003). Currently, the most effective therapy for alleviating the symptoms of PD is levodopa (L-DOPA) (Fahn and Przedborski, 2000), which increases the levels of dopamine in the brain. Although, there is no evidence that L-DOPA alters the progression of the disease, on one hand, speculations exist that L-DOPA may actually contribute to the progression of PD (Fahn, 1997; Weiner, 2000) while on the other, it is thought that L-DOPA is actually neuroprotective (Rajput, 2001) and non-toxic to the human substantia nigra (Rajput, 2001). For reasons that are not yet understood, dopaminergic neurons in the SNpc appear to be more susceptible to damage than other neurons in the brain. Theories as to why this situation exists include genetics (Vila and Przedborski, 2004), excitotoxicity (Olanow and Tatton, 1999), inflammation in

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the brain due to changes in the neuronal environment (Langston *et al.*, 1999; Hunot and Hirsch, 2003, Teismann *et al.*, 2003), protein aggregation (Ii *et al.*, 1997; Trojanowski *et al.*, 1998) oxidative stress, (Fahn and Cohen, 1992; Przedborski and Jackson-Lewis, 2000), and growth factor (neurotrophin) decline (Mogi *et al.*, 1999; Nagatsu *et al.*, 2000).

About 10% of the PD cases are familial. To date, a number of genetic mutations have been found both in multiple pedigrees and in single families. Multiple pedigree mutations include those found in the alpha-synuclein, parkin, Nurr-1 (nuclear receptor related-1) and DJ-1 genes whereas UCHL-1 (ubiquitin c-terminal hydrolase-1) and NF-M (neurofilament medium) gene mutations have been localized to single families (Huang *et al.*, 2004). Most, if not all of these identified genes function for the survival of the dopamine neuron (synthesis, metabolism, energy supply, cellular detoxification). Thus, any mutation in these genes could lead to misfunctions in the dopamine neuron making them more susceptible to such problems as energy crisis and oxidative stress that could lead to eventual death. In addition to these PD-inducing mutations, several mechanisms have been proposed regarding the etiology of PD. These include ion homeostasis, neuroinflammation, protein aggregation and alterations in growth factors.

Calcium homeostasis is important to normal dopamine neuron function. The NMDA (*N*-methyl-D-aspartate) and the AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors, both ionotrophic glutamate receptors, are, in part, responsible for intracellular calcium homeostasis (Rego and Oliveira, 2003). Overstimulation of these glutamate receptors can alter local calcium homeostasis. Calcium is known to up-regulate enzymes like phospholipase A_2, nitric oxide synthase and xanthine oxidase, all of which are found in mitochondria and all can stimulate reactive oxygen species (ROS) production. Thus, if local calcium control is compromised, resulting in an excitatory-stimulated release of ROS and if existing antioxidant systems cannot handle the produced ROS, mitochondrial dysfunction and damage to several synaptic and intracellular proteins ensues.

Progression of a number of neurological diseases has been shown to be related to inflammation in the brain, which can affect the neuronal environment. For instance, multiple sclerosis is a neuroinflammatory disease that causes a loss of the myelinated tracts in the CNS (Hafler, 2004), and recent evidence has shown that there is an inflammatory component to amyotrophic lateral sclerosis (Drachman *et al.*, 2002). Furthermore, supporting a role for inflammation in PD is the finding by Langston and colleagues that brains from individuals, who died from a PD-like syndrome resulting from the self-administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and who lived for 3-16 years following exposure, showed a significant presence of activated microglia (Langston *et al.*, 1999). However, inflammation, thus far, has not been shown to be the cause of PD but rather it is suggested that inflammation may be instrumental in its progression.

Although there is still much debate on the subject, the finding of protein aggregates (both intracellular and extracellular) in many of the neurodegenerative diseases including PD has led to the hypothesis that the improper disposition of proteins may be toxic to the dopamine neuron and can contribute to the neurodegenerative process. In the simplest of terms, the ubiquitin-proteasome system (UPS) is like a sink which degrades both abnormal and damaged proteins in the neuron. Proteins are first ubiquinated by the covalent attachment of a polyubiquitin chain and then the whole complex is degraded by the 26S proteasome (Vigouroux *et al.*, 2004). If this system fails to operate properly, it is thought that the aggregation of proteins to be disposed of follows. For example, Lewy bodies are a pathological hallmark of PD and they contain significant amounts of modified alpha-synuclein (Dauer and Przedborski, 2003). Recent reports have shown that aggregated alpha-synuclein not only binds to but also inhibits ubiquitin-dependent proteasomal function (Snyder *et al.*, 2003). Furthermore, oxidized proteins can accumulate in the neuron and this abnormal accumulation of proteins may be toxic enough to put the neuron in an oxidative stress situation which is a highly damaging event.

The growth factor decline hypothesis begs the question as to why these substances are decreased in the SNpc of PD brains. Growth factors (neurotrophins) are proteins that are normally highly expressed in the substantia nigra (SN) and several lines of evidence demonstrate a decrease in growth factors, particularly glial-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) in the SN of PD brains (Chauhan *et al.*, 2001). The reason for these decreases remains unknown. And, there is nothing known about growth factor decline and oxidative stress. What is clear is that most of the aforementioned hypotheses involve some kind of oxidative stress situation. We and others have used MPTP to follow the oxidative stress hypothesis and the proposed roles of superoxide, nitric oxide and dopamine in the vulnerability of the dopamine neuron (FIG. 1).

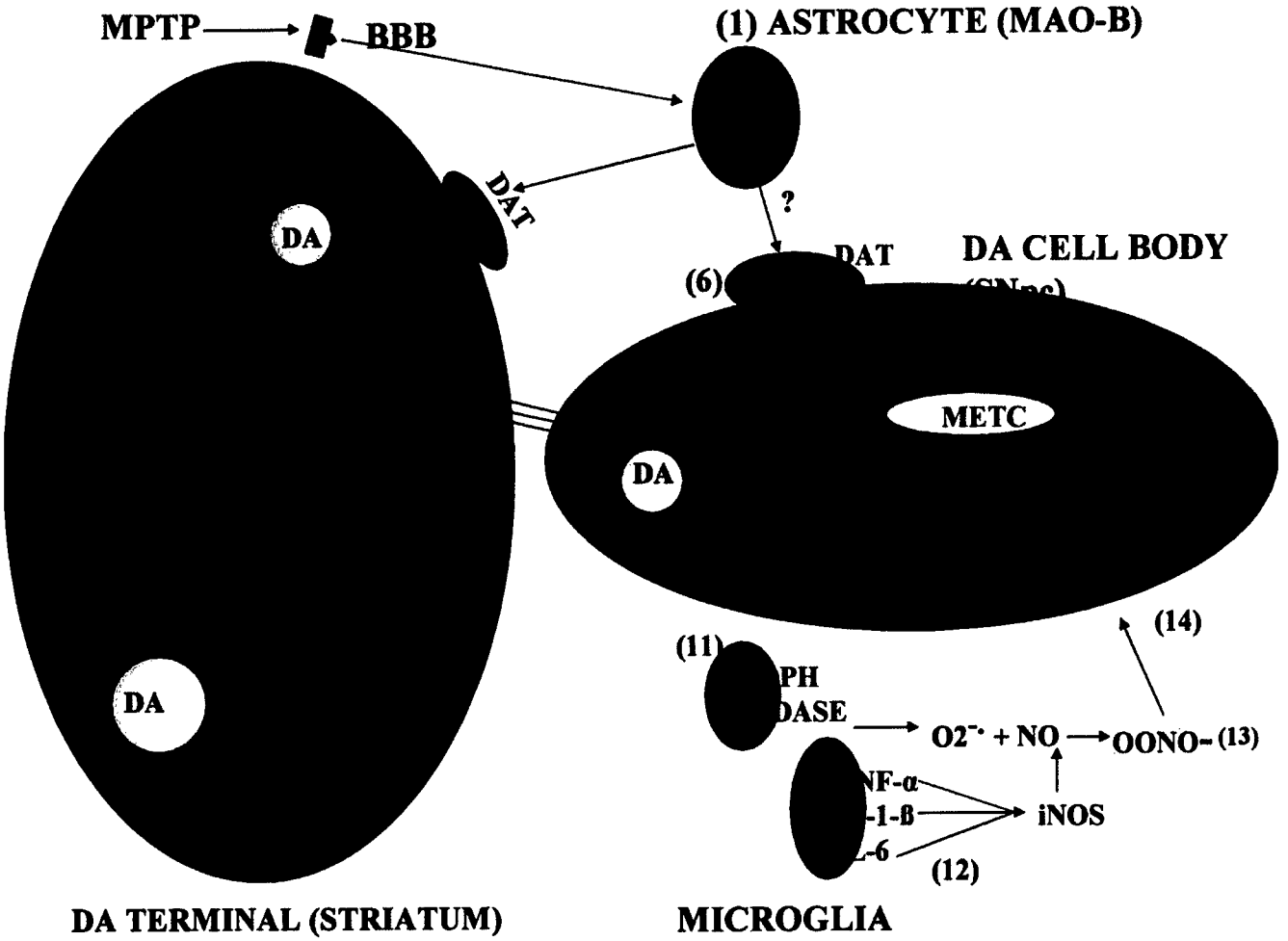

FIGURE 1 Proposed MPTP action in the nigrostriatal dopaminergic pathway. After systemic administration, MPTP freely crosses the blood-brain barrier and is taken up into astrocytes where it is metabolized by monoamine oxidase-B (MAO-B) to MPP^+ (1). It then exits the astrocyte by an unknown mechanism and is taken up by the dopamine transporter (DAT) into the dopamine terminals in the striatum. There, MPP^+ is sequestered into the storage vescicles (2) and in the process pushes out dopamine (3) which is oxidized to either dopamine-*o*-quinone (dopamine-*o*-Q), 6-hydroxydopamine (6-OHDA), 3,4-dihydroxyphenylacetaldehyde (DOPAL) or 5-cysteinyldopamine (5-cysteinyl- dopamine) (4). Dopamine-*o*-Q (DA-*o*-Q), 6-OHDA and DOPAL have all been shown to be toxic and can attack dopamine nerve terminals (5) that lead back to the SNpc dopamine cell body. In the meantime, in the extracellular space in the SNpc, MPP^+ that is extruded from astrocytes, is taken up into the neuron by the DAT (6). Once in the cell body (7), MPP^+ blocks the complex 1 site of the mitochondrial electron transport chain (METC) which causes the METC to kick out the superoxide radical. Neuronal nitric oxide synthase (nNOS) in the METC membrane up-regulates, which increases the presence of nitric oxide (NO). In the cytosol of the dopamine neuron, superoxide and NO interact to produce the strong oxidant peroxynitrite ($OONO^-$) (8) which can damage cellular proteins, lipids and DNA. Dopamine in the neuron is oxidized to dopamine-*o*-Q and DOPAL (9) or can be released and subjected to hydroxyl radical attack (6-OHDA?) (10) which can also damage the neuron. Once the neuron is damaged, an inflammatory response ensues in which microglia in the extracellular space become activated. During the activation process, certain enzymes are up-regulated [NADPH oxidase (11), tumor necrosis factor (TNF)-α, interleukin-1-beta (IL-1-ß), IL-6 and inducible NOS (iNOS) (12)] which stimulate superoxide and NO production. These two then react to produce $OONO^-$ (13) which can attack the neuronal membrane (14).

The MPTP Neurotoxic Process

As a highly lipophilic compound, MPTP can be absorbed through the skin, be ingested, injected, and snorted. However administered, MPTP rapidly crosses the blood brain barrier and is taken up into glial cells by monoamine (Brooks *et al.*, 1989) and glutamate transporters (Hazell *et al.*, 1997) or pH-dependent antiporters (Kopin *et al.*, 1992; Marini *et al.*, 1992). Once inside glial cells, MPTP is converted to $MPDP^+$ (1-methyl-4-phenyl-2,3-dihydropyridinium) by monoamine oxidase-B (MAO-B) and then to MPP^+ (1-methyl-4-pyridinium) (Ransom *et al.*, 1987) by spontaneous oxidation. Since MPP^+ is a polar compound, it cannot cross membranes; it is speculated that MPP^+ is extruded from glia via some kind of transport system. Evidence for a role of glia in the conversion of MPTP

to MPP^+ comes from Brooks *et al.* (1989) who demonstrated that fluoxetine, a serotonin uptake inhibitor, attenuates MPTP-induced dopaminergic toxicity but does not interfere with MPTP metabolism. Following extrusion into the extracellular space, MPP^+ is taken up into the dopamine neuron by the dopamine transporter (DAT) (Kostic *et al.*, 1996). This transporter may be damaged in the MPP^+ uptake process, as recent evidence by Jakowec *et al.* (2004) have shown that the number of DAT in the SN following MPTP administration is decreased. MPTP targets primarily dopamine neurons and the syndrome it produces, over a period of about a week (Jackson-Lewis *et al.*, 1995), is reminiscent of end-stage PD. MPTP causes a far greater loss of dopamine neurons in the SNpc than of those dopamine neurons in the ventral tegmental area. It also produces about 90% degeneration of dopamine nerve terminals in the striatum (Jackson-Lewis *et al.*, 1995). In order for this level of damage to occur in the nigrostriatal dopaminergic pathway, MPP^+ has to stimulate or recruit dopamine-related metabolites from within this pathway.

MPTP and Glial Cells

The non-neuronal support system in the CNS are the glial cells (Abbott, 1988). Under physiological conditions, glia secrete substances into the extracellular environment that support the normal functioning of the neuron (Abbott, 1988). For instance, not only is it known that microglia remove debris from the neuronal environment but, depending on the situation, they can be a source of neurotrophic and neuroprotective molecules such as interleukin-6, basic fibroblast growth factor, epidermal growth factor, insulin-like growth factor and epidermal growth factor. On the other hand, they can also produce neurotoxic compounds like nitric oxide, superoxide, tumor necrosis factor, glutamate, arachidonic acid and proteolytic enzymes (Banati *et al.*, 1993). Astrocytes seem to exert a protective effect on dopaminergic neurons, as it has been demonstrated that they can produce neurotrophins like nerve growth factor, ciliary growth factor and interleukin-6 (Muller *et al.*, 1995) as well as GDNF (Bohn, 1999). What is extremely interesting about these glial cells is that they may represent a double-edged sword when it comes to MPTP, for in the MPTP neurotoxic process, it is in glia that MPTP is metabolized to MPP^+ by MAO-B, and microglia produce molecules such as the superoxide radical and nitric oxide which are toxic to dopamine neurons.

MPTP, Superoxide and the SNpc Environment

The environment surrounding SNpc neurons can control the fate of these cells. For example, following MPTP administration, both the extracellular and the intracellular environments of the SNpc dopamine neuron are altered in such a way that they are no longer part of a supportive system but rather contain detrimental components. Our early studies using transgenic mice that overexpress the copper-zinc form of superoxide dismutase (CuZnSOD) and that were treated with MPTP show that the SNpc of these mice was protected against the damaging effects of MPTP (Przedborski *et al.*, 1992), thus implying the involvement of the superoxide radical. Furthermore, Wu *et al.* (2003) have shown that the infusion of SOD1 into the striatum of MPTP-treated mice is neuroprotective to SNpc neurons, which defines a role for the superoxide radical in the MPTP neurotoxic process. Since CuZnSOD is an extracellular enzyme (Fridovich, 1995), these results suggest that the extracellular environment of the dopamine neuron is perturbed or altered by the superoxide radical early in the neurotoxic process.

A significant source of the superoxide radical in the extracellular environment is NADPH oxidase (Gao *et al.*, 2003; Wu *et al.*, 2003). NADPH oxidase is a multimeric microglial enzyme that is composed of a number of subunits that include $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$, and $p40^{phox}$ (Babior, 1999). In resting microglia, this enzyme is inactive because $gp91^{phox}$ and $p22^{phox}$ are separated from the other phox subunits. However, following MPTP administration to mice, the NADPH oxidase complex within the microglia becomes activated because the p47 phox subunit is phosphorylated followed by the movement of the whole complex to the microglial membrane where it assembles with $gp91^{phox}$ and $p22^{phox}$. This makes the NADPH oxidase complex able to stimulate the production of the superoxide radical. Wu *et al.* (2003), using hydroethidium injections in MPTP-treated mice, visualized the presence of the superoxide radical within microglia located in the SNpc environment of these mice. Up-regulation of NADPH oxidase in postmortem SNpc tissues from PD brains was also shown (Wu *et al.*, 2003). The superoxide radical is then extruded into the extracellular environment where its presence not only alters the neuronal environment but also stimulates the production of secondary oxidants (Babior, 1999) which can, in turn, influence the integrity of the dopamine neuronal membrane, enter the dopamine neuron and affect its internal environment.

Dopamine neurons, as abundant as they are in the SNpc, are likely a victim of their own environment. Once MPP^+ exits the glial cells, it is taken up from the extracellular space into the dopamine neuron via the DAT (Javitch *et al.*, 1985; Bezard *et al.*, 1999).

Although recent evidence shows that these transporters are injured during the uptake process (Jakowec *et al.*, 2004), enough of them remain to transport MPP^+ into the cytosol of the dopamine neuron. DAT are absolutely necessary for the MPTP neurotoxic process as several groups (Gainetdinov *et al.*, 1997; Bezard *et al.*, 1999) have shown that MPTP does not harm mice lacking DAT. In the cytosol of the dopamine neuron, when MPP^+ is not taken up into the vesicles, MPP^+ can assist in altering the internal environment of the dopamine neuron by blocking the mitochondrial electron transport chain (METC) at the complex I site (Nicklas *et al.*, 1985; 1987). The major organelle within the dopamine neuron that produces the lion's share of superoxide radicals is the mitochondrion (Beal, 2003). This organelle controls oxidation-reduction reactions and is a major source of cellular energy through its oxidative phosphorylation reactions (Przedborski and Jackson-Lewis, 2000). At the complex I site of the METC, the superoxide radical is released into the cytosol where, under physiological conditions, it is controlled by the manganese form of SOD (MnSOD), which is located in the internal membrane of the mitochondrion (Keller *et al.*, 1998). Many investigators have found a decrease in complex I in various tissues including brain tissue from PD patients (Mizuno *et al.*, 1989; Shapira, 1990). Thus, low activity of complex I in the METC translates to increased production of superoxide radicals, a depletion of MnSOD and an oxidative stress within the dopamine neuron. An overabundance of superoxide radicals, as stimulated by the presence of MPP^+, apparently can no longer be controlled by MnSOD. Klivenyi and colleagues (Klivenyi *et al.*, 1998) have shown that, as long as sufficient stores of MnSOD are present: 1) mice are protected against the damaging effects of MPTP; and 2) the superoxide radical influences the internal environment of the dopamine neuron. Furthermore, MPP^+ has also been shown to affect complex III (Mizuno *et al.*, 1988), such that the increased production of the superoxide radical here also contributes to the disruption of the normal cytosolic environment within the SNpc dopamine neuron. The relevance of this particular scenario to PD is not well understood because it is not clear whether the deficit in complex I is or is not a cause of PD.

MPTP, Nitric oxide and the SNpc Environment

Nitrative stress related to NO has been documented in PD brains through demonstration of the presence of the inducible form of nitric oxide synthase (iNOS) (Hunot *et al.*, 1996; 1999) and has been tied, in part, to the activated glia in the vicinity of SNpc dopamine neurons. Evidence of the up-regulation of iNOS in glia following MPTP administration points to an indirect process rather than a direct up-regulation of this enzyme (Ciesielska *et al.*, 2003). In glia within the area of the SNpc and the striatum, MPP^+ stimulates the up-regulation of proinflammatory cytokines such as TNF-α, interleukin-1-beta (IL-1ß) and interleukin-6 (IL-6) (Youdim *et al.*, 2002; Teismann *et al.*, 2003) in a time-dependent manner (Hebert *et al.*, 2003) as early as 12-18 hours prior to the induction of iNOS (Hunot *et al.*, 1999). Immunohistochemical studies (Liberatore *et al.*, 1999; Dehmer *et al.*, 2000) show that iNOS up-regulation occurs in microglia 24 hours after the administration of MPTP, which suggests that the proinflammatory cytokines may stimulate the up-regulation of the iNOS enzyme and thereby increase the production of NO within the glia. In a personal communication, Wu and Przedborski (Personal Communication) noted that endothelial NOS (eNOS) is found in the brain vasculature and does not contribute to the MPTP neurotoxic process. In contrast, neuronal NOS (nNOS), shown to be decreased within non-dopamine neurons (? interneurons) in the SNpc following MPTP administration (Watanabe *et al.*, 2004), probably does contribute to the MPTP neurotoxicity in the SNpc. Furthermore, since nNOS knockout mice were only partially protected against the damaging effects of MPTP and 7-nitroindazole, a selective inhibitor of nNOS that has little if any cardiovascular effects, offered a greater but not a total protection (Przedborski *et al.*, 1996), it is likely that nNOS is a contributor to NO presence in the extracellular space and to the alterations in the extraneuronal environment of the dopamine neurons in the SNpc.

NO is not a free radical, is highly lipophilic, can readily traverse membranes without the need of a transport system and has the ability to travel as far as 300 microns from its site of production (Lancaster, 1996). Under physiological conditions, both nNOS and iNOS produce significant amounts of NO that are ever present in the extracellular space while levels of the superoxide radical, constantly produced in many biological reactions within the brain, are kept in check by the abundance of SOD. In the pathology of PD and in the MPTP model, increased amounts of the superoxide radical and NO are pushed into the extracellular milieu surrounding the dopamine neuron. Here, they can react with each other at a faster rate than the superoxide radical can be dismutated by the extracellular CuZnSOD to produce the most damaging secondary oxidant peroxynitrite (Przedborski *et al.*, 2000). Peroxynitrite can damage neuronal membrane proteins and lipids (Przedborski *et al.*, 2000). Thus, the extracellular neu-

ronal environment of SNpc is disturbed or compromised and is no longer supportive for the dopamine neuron either in PD or in the MPTP model.

Although the superoxide radical does disturb the internal environment of the dopamine neuron, it is, by itself, not overwhelmingly toxic. In the internal milieu of the SNpc dopamine neuron, aside from affecting the METC, MPP^+ has been demonstrated to increase the expression of the cyclooxygenase-2 (COX-2) enxyme (Teismann *et al.*, 2003). COX-2 is the rate-limiting enzyme in the conversion of arachidonic acid to PGH_2 which is then further metabolized to PGE_2 (O'Bannion, 1999). The NO present in the SNpc dopamine neuron following MPTP administration most likely enters the dopamine neuron after having traveled some distance from its non-dopamine neurons in the SNpc that contain nNOS. When both the superoxide radical and NO are in excess in the internal milieu of the dopamine neuron after MPTP exposure, PGE_2 catalyzes the reaction between these two relatively mildly toxic compounds to produce the secondary oxidant peroxynitrite (Ischiropoulos and al-Mehdi, 1995; Przedborski and Vila, 2003) which again creates a severely hostile environment for the dopamine neuron. Peroxynitrite nitrates internal cellular components such as enzymes, fatty acids, proteins, lipids, amino acids and DNA (Radi *et al.*, 2002) of which one of the most important of these is the tyrosine hydroxylase (TH) enzyme. This enzyme is the rate-limiting enzyme in the synthesis of dopamine and is either down-regulated or damaged in PD and in the MPTP model such that the production of dopamine is severely compromised (Ara *et al.*, 1997).

Dopamine Toxicity and the SNpc Environment

The dopamine neuron in the SNpc may indeed be, at least in part, a contributor to its own death. Following MPTP administration, huge amounts of dopamine are released from intracellular stores into the extracellular space (Lau *et al.*, 1991; Schmidt *et al.*, 1999). Once released, dopamine is either enzymatically metabolized by monamine oxidase-B to 3,4-dihydroxyphenylacetic acid and in the process, the hydroxyl radical is kicked out (Burke *et al.*, 2004) or it auto-oxidizes to form a number of toxic compounds including 6-hydroxydopamine (Graham, 1978). 6-hydroxydopamine is a known neurotoxin that has been used extensively for animal models in PD research (Jeon *et al.*, 1995; Przedborski *et al.*, 1995). It has been demonstrated that this compound destroys striatal dopamine terminals which results in the death of SNpc dopamine neurons (Przedborski *et al.*, 1995). Interestingly, one of the findings in PD and in the MPTP model is that there is a greater loss of striatal dopamine nerve terminals than dopamine cell bodies in the SNpc (Fahn and Przedborski, 2000). This may be related to the huge release of dopamine from the storage vesicles caused by the uptake of MPP^+. Furthermore, although 6-hydroxydopamine has never been found in brain tissues from PD patients nor in brains from the MPTP model, one can speculate on the possibility that 6-hydroxydopamine or a similarly related compound may contribute negatively to the external environment that surrounds the dopamine neuron, since dopamine is susceptible to hydroxyl radical (secondary oxidant) attack (Cohen, 1984). A more interesting scenario, however, has been proposed with 3,4-dihydroxyphenylacetaldehyde (DOPAL). DOPAL is the intermediate dopamine metabolite that has been shown to be neurotoxic (Burke *et al.*, 2003). To demonstrate that it is DOPAL and not dopamine that is neurotoxic, Burke and colleagues (Burke *et al.*, 2003) injected varying concentrations of both compounds into the SNpc of rats. These researchers showed that DOPAL was 5-10 times more neurotoxic than dopamine. Thus, in the extracellular space, because MAO-B metabolizes dopamine to DOPAL (Fornai *et al.*, 2000; Burke *et al.*, 2004), dopamine via DOPAL, possibly contributes to changes in the extracellular milieu. DOPAL may also be the reason why dopamine terminals are severely damaged.

In the internal metabolism of the DA neuron, DA can be oxidized to dopamine-*o*-quinone and further to 5-cysteinyldopamine (Hastings, 1995). Aside from having a role in peroxynitrite formation through its stimulation of PGE_2, the COX-2 enzyme can facilitate the oxidation of dopamine which can damage protein-bound sulfhydryl groups (Hastings, 1995). Using HPLC analysis, Teismann *et al.* (2003) showed that MPTP administration elevates ventral midbrain 5-cysteinyldopamine, which is considered a stable modification of dopamine and evidence that the formation of dopamine-*o*-quinone has occurred. Dopamine-*o*-quinone can contribute to the upheaval of the internal neuronal environment through glutathione depletion and the inactivation of TH (Kuhn *et al.*, 1999). On the other hand, while dopamine is metabolized to DOPAL extraneuronally by MAO-B, within the neuron, DOPAL is formed by MAO-A (Burke *et al.*, 2004). Furthermore, DOPAL is the major metabolite of dopamine in the human brain (Burke *et al.*, 1999) and levodopa, the drug of choice in the treatment of PD, has been shown to elevate significantly levels of DOPAL in the brain (Fornai *et al.*, 2000). As stated earlier, DOPAL has been shown to destroy the dopamine neuron at concentrations much lower than dopamine

itself (Burke *et al.*, 2003). Whether MPTP can elevate DOPAL levels in the brain and mimic the death of dopamine nerve terminals as seen in PD remains to be determined.

CONCLUSIONS

Environment plays a significant role in the well-being of the dopamine neuron. Several cell types including glia and the compounds that these cells secrete work together to maintain an environment suitable for dopamine neuron survival. Yet, at the same time, these same cells and agents, when perturbed such as following MPTP administration, can contribute to the death of the dopamine neuron through reactions which alter their physiological concentrations in the SNpc, thus putting the dopamine neuron in a compromised (oxidative stress) situation. Interestingly, the major players in both environments are relatively the same as is their interplay. Thus, dopamine, superoxide and nitric oxide may all conspire to keep the dopamine neuron in a highly sensitive state, and when presented with a catalyst like MPTP, this sensitivity can shift to vulnerability.

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APPENDIX 16

Neurobiology of Disease

Ablation of the Inflammatory Enzyme Myeloperoxidase Mitigates Features of Parkinson's Disease in Mice

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Parkinson's disease (PD) is characterized by a loss of ventral midbrain dopaminergic neurons, which can be modeled by the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Inflammatory oxidants have emerged as key contributors to PD- and MPTP-related neurodegeneration. Here, we show that myeloperoxidase (MPO), a key oxidant-producing enzyme during inflammation, is upregulated in the ventral midbrain of human PD and MPTP mice. We also show that ventral midbrain dopaminergic neurons of mutant mice deficient in MPO are more resistant to MPTP-induced cytotoxicity than their wild-type littermates. Supporting the oxidative damaging role of MPO in this PD model are the demonstrations that MPO-specific biomarkers 3-chlorotyrosine and hypochlorous acid-modified proteins increase in the brains of MPTP-injected mice. This study demonstrates that MPO participates in the MPTP neurotoxic process and suggests that inhibitors of MPO may provide a protective benefit in PD.

Key words: MPTP; Parkinson's disease; oxidative stress; inflammation; neuroprotection; nitrotyrosine

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by disabling motor abnormalities, which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (Dauer and Przedborski, 2003). Its main neuropathological feature is the loss of the nigrostriatal dopaminergic neurons, the cell bodies of which reside in the substantia nigra pars compacta (SNpc) and nerve terminals of which extend to the striatum (Dauer and Przedborski, 2003). Except for a handful of inherited cases related to known gene defects (Vila and Przedborski, 2004), PD is a sporadic condition of unknown pathogenesis (Dauer and Przedborski, 2003). However, epidemiological studies suggest that inflammation increases the risk of developing PD (Chen et al., 2003), and experimental models of PD show that inflammatory oxidants modulate SNpc dopaminergic neuronal death (Liberatore et al., 1999; Gao et al., 2002; Wu et al., 2002, 2003). For instance, NADPH oxidase and inducible nitric oxide synthase (iNOS), which are major sources of inflammatory oxidants, are upregulated in damaged areas in both PD and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD (Hunot et al., 1996; Liberatore et al., 1999; Wu et al., 2003). Studies of mice deficient in NADPH oxidase or iNOS indicate that superoxide radical (O_2^-) and NO contribute to the MPTP-induced neurodegenerative process (Liberatore et al., 1999; Wu et al., 2003). However, both O_2^- and NO are relatively unreactive, and a variety of secondary oxidants, such as peroxynitrite ($ONOO^-$), are more likely to account for the injurious capacity of inflammation in PD. Supporting this view are the demonstrations that levels of 3-nitrotyrosine, a major product of $ONOO^-$ oxidation of proteins, are elevated in affected brain areas after MPTP injections to mice (Pennathur et al., 1999), for the most part in an iNOS-dependent manner (Liberatore et al., 1999).

Levels of *O,O′*-dityrosine also increase markedly in the SNpc of MPTP-intoxicated animals (Pennathur et al., 1999). This is an intriguing finding because *O,O′*-dityrosine is a relatively minor product of $ONOO^-$ (Pennathur et al., 1999). Conversely, myeloperoxidase (MPO), and not $ONOO^-$, seems to promote *O,O′*-dityrosine formation in this model of PD (Pennathur et al., 1999). Moreover, MPO can use the NO degradation product NO_2^- to generate reactive nitrogen species (RNS) (van der Vliet et al., 1997), and studies of mice deficient in MPO demonstrate that this enzyme is one of the major sources of 3-nitrotyrosine during acute inflammation (Gaut et al., 2002). Thus, these results raise the unanticipated possibility that MPO, a heme enzyme expressed in abundance in a variety of phagocytic cells (Hampton et al., 1998), would contribute to the MPTP-induced neurodegenerative process and would represent a previously unrecognized culprit in the inflammatory-mediated oxidative insult associated with diseases such as PD. Consistent with this hypothesis, we

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show here not only that MPO is detected in affected brain areas of MPTP-injected mice and PD patients, specifically in glial cells, but also that mutant mice deficient in MPO are more resistant to MPTP-induced dopaminergic neurotoxicity. These findings indicate that MPO does participate in the MPTP neurotoxic process and suggest that inhibitors of MPO may provide protective benefit in PD.

Materials and Methods

Animals and treatment. Procedures using laboratory animals were in accordance with the National Institutes of Health (NIH) guidelines for the use of live animals and approved by the Institutional Animal Care and Use Committee of Columbia University. The mice used in this study were 10-week-old male C57BL/6J mice (Charles River Laboratories, Wilmington, MA) and MPO-deficient mice that had been backcrossed >10 times into the C57BL/6J background (Brennan et al., 1985) and their wild-type (WT) littermates, all weighing 22–25 g. For MPTP intoxication, 4–10 mice per group received four intraperitoneal injections every 2 h of MPTP–HCl (18–20 mg/kg of free base; Sigma-Aldrich, St. Louis, MO) dissolved in saline. Mice were killed from 0–7 d after the last injection, and their brains were used for morphological and biochemical analyses. Control mice received saline only. MPTP handling and safety measures were in accordance with published guidelines (Przedborski et al., 2001).

RNA extraction and reverse transcription-PCR. Total RNA was extracted from selected brain regions and at selected time points after MPTP and used for reverse transcription-PCR analysis as described previously (Wu et al., 2003). The primers used for mouse MPO and β-actin were as follows: MPO, 5′-AGGATAGGACTGGATTGCCTG-3′ (forward) and 5′-GTGGTGATGCCAGTGTTGTCA-3′ (reverse); β-actin, 5′-CTTTGATGTCACGCACGATTTC-3′ (forward) and 5′-GGGCCGCTCTAGGCACCAA-3′ (reverse). The thermal cycling conditions of the PCR were 94°C for 3 min, followed by 23–35 cycles for 20 s at 94°C, 1 min at 60°C, 1 min at 72°C, and a final extension at 72°C for 5 min. After amplification, products were separated on a 5% PAGE and quantified by a FluorChem 8800 digital image system (Alpha Innotech, San Leandro, CA). PCR products were of expected sizes, and sequences were confirmed by direct cycle sequencing.

Immunoblots. Mouse brain protein extracts from selected regions were prepared and used for Western blot analysis as described previously (Wu et al., 2003). The primary antibodies used were as follows: a rabbit polyclonal antibody raised against a 14 aa peptide representing the C terminus of the mouse MPO (NTLPKLNLTSWKET; 1:1000 dilution; generated by J.W.H.'s laboratory) and a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma, St. Louis, MO). A horseradish-conjugated secondary antibody (1:500–1:25,000; Amersham Biosciences, Piscataway, NJ) and a chemiluminescent substrate (SuperSignal Ultra; Pierce, Rockford, IL) were used for detection. Bands were quantified using the FluorChem 8800.

MPO isolation and activity. The methods used to prepare brain samples and to measure MPO activity are slight modifications of those described previously by Daugherty et al. (1994). In brief, fresh mouse tissues from selected brain regions were homogenized in a 100 mM sodium phosphate buffer, pH 7.0, containing 1% (wt/vol) cetyltrimethylammonium bromide (CTAB) and centrifuged (6000 × *g*, 4°C, 10 min). Then 1 mM $CaCl_2$, $MnCl_2$, and $MgCl_2$ (final concentration) were added to each sample before being incubated overnight at 4°C with 0.3 ml of concanavalin A-Sepharose B (Sigma). The gel was then pelleted by centrifugation and washed three times with a 0.1 M sodium acetate buffer, pH 6.0, containing 0.1 M NaCl and 0.05% CTAB. Then samples were centrifuged (6000 × *g*, 5 min) to remove residual washing buffer. The glycoprotein bound to the lectin gel was then eluted by incubation with 0.15 ml elution buffer (0.5 M methyl α-D-mannoside in washing buffer) for 30 min. After the last centrifugation, final supernatants were collected and used immediately to assess MPO activity by monitoring the oxidation of tetramethylbenzidine as described previously (Andrews and Krinsky, 1982). The absorbance was read at 655 nm with a microplate reader (Bio-Rad, Hercules, CA).

Mouse MPO, glial fibrillary acidic protein, β_2 integrin MAC-1 (CD116/CD18), neutrophil, and tyrosine hydroxylase immunohistochemistry. At selected time points after MPTP, mice were killed, and their brains were processed for immunohistochemical studies following our standard protocol for single or double immunostaining (Wu et al., 2003). The primary antibodies used were rabbit polyclonal anti-MPO (1:500; Lab Vision, Fremont, CA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; 1:500; Chemicon, Temecula, CA), mouse monoclonal anti-MAC-1 (1:1000; Serotec, Raleigh, NC), and the monoclonal rat anti-mouse neutrophil antibody MCA771GA (1:100; Serotec). Immunostaining was visualized by 3,3′-diaminobenzidine (DAB) or fluorescein and Texas Red (Vector Laboratories, Burlingame, CA) and examined by regular or confocal microscopy. Colocalization studies were performed on doubly immunofluorescent stained sections, which were analyzed with an LSM 510 META laser-scanning microscope (Zeiss, Thornwood, NY).

For quantitative tyrosine hydroxylase (TH) immunostaining, mice were killed 7 d after MPTP. Both striatal and nigral sections (30 μm), spanning the entire extent of the structures, were incubated with a polyclonal anti-TH antibody (1:1000; Calbiochem, San Diego, CA) for 48 h at 4°C. Immunoreactivity was visualized by incubation in DAB, glucose, and glucose oxidase, and sections were counterstained with thionin. The total numbers of TH- and Nissl-positive neurons in the SNpc were counted stereologically using the optical fractionator method (West, 1993) as used previously (Tieu et al., 2003). Striatal OD of TH immunostaining, determined by the Scion (Frederick, MD) Image program, was used as an index of striatal density of TH innervation (Tieu et al., 2003). The concentration of anti-TH antibody and DAB used here and the length of time striatal sections were incubated in DAB were the same as reported previously (Tieu et al., 2003).

Human samples. All human samples were obtained from the New York Brain Bank at Columbia University (http://cumc.columbia.edu/research/equip/eq-tb_bb.htm). Procedures using this autopsy material were in accordance with the NIH guidelines for human studies and approved by the Institutional Review Board of Columbia University. Samples used in this work included the cerebellum, striatum, and ventral midbrain (for PD and controls); the caudate nucleus [for Huntington's disease (HD) and controls]; and the frontal motor cortex [for amyotrophic lateral sclerosis (ALS) or motor neuron disease and controls]. All of these cases were selected on the basis of neuropathological diagnoses using the criteria for definite PD, HD, and ALS outlined in the supplemental material (available at www.jneurosci.org). Relevant clinical and neuropathological information regarding all of the cases used here are presented in supplemental Table 2 (available at www.jneurosci.org as supplemental material). The procedures for Western blot analysis and immunohistochemistry in human tissues were identical to those described above in mouse tissues; the primary anti-MPO antibody was a rabbit anti-human MPO antibody (DakoCytomation, Carpinteria, CA) used at 1:1000 for Western blot and 1:200 for immunohistochemistry, as well as a rabbit polyclonal anti-GFAP antibody (1:10,000; DAKO, Carpinteria, CA). Visualization of the bound antibody was achieved using chromogenes SG (blue/gray) and 3-amino-9-ethylcarbazole (red) from Vector Laboratories.

MPTP metabolism. Striatal 1-methyl-4-phenylpyridinium (MPP^+) levels were determined by HPLC with UV detection (λ = 295 nm) in WT and MPO-deficient mice at 90 min after the last injection of 20 mg/kg MPTP. Striatal tissue lactate production induced by MPP^+ and synaptosomal uptake of [^{3}H]MPP^+ were performed as described previously (Wu et al., 2003). The assays were repeated three times, each time using duplicate samples.

Mass spectrometric analysis. At selected time points, anesthetized mice were perfused with ice-cold 50 mM sodium phosphate, pH 7.4, containing an antioxidant mixture made of 100 μM diethylenetriaminepentaacetic acid, 1 mM butylated hydroxytoluene, 10 mM 3-amino-1,2,4-triazole, and 1% ethanol (v/v) to minimize *ex vivo* oxidation. The ventral midbrain and cerebellum were then dissected and pulverized in liquid N_2, delipidated, dialyzed to remove low-molecular weight compounds, and hydrolyzed using HBr instead of HCl to prevent artifactual chlorination. [$^{13}C_6$]-Ring-labeled internal standards were added before hydrolysis. The amino acids were isolated using a C-18 solid-phase extraction col-

umn and subjected to derivatization and analysis by isotope dilution gas chromatography/mass spectroscopy (GC/MS) (Heinecke et al., 1999).

Detection of hypochlorous acid-modified protein. Immunochemical detection of hypochlorous (HOCl)-modified proteins was performed with the antibody HOP-1 (clone 2D10G9; dilution 1:500; provided by E. Malle, Medical University of Graz, Graz, Austria). HOP-1 is specific for HOCl-modified epitopes/proteins and does not cross-react with other oxidative modifications (Malle et al., 1995; Hazell et al., 1996). Immunostaining was visualized by using DAB, and sections were counterstained with methylgreen (Vector Laboratories).

Statistical analysis. All values are expressed as mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pairwise comparisons between means were tested by Newman–Keuls *post hoc* testing. In all analyses, the null hypothesis was rejected at the $p \leq 0.05$ level.

Results

MPO is induced in the mouse ventral midbrain during MPTP-induced dopaminergic neurodegeneration

To examine the possibility that MPO is a component of the inflammatory response seen in the MPTP model of PD (Liberatore et al., 1999; Wu et al., 2002), we first assessed MPO mRNA and protein content in the ventral midbrain (i.e., brain region containing the SNpc dopaminergic neurons) over the entire active phase of neurodegeneration and gliosis provoked by this neurotoxin (Liberatore et al., 1999; Przedborski and Vila, 2001). In saline-injected control mice, the ventral midbrain contained low levels of MPO mRNA and protein (Fig. 1*A–C*). In contrast, in MPTP-injected mice, ventral midbrain levels of both MPO mRNA and protein increased in a time-dependent manner (Fig. 1*A–C*). Ventral midbrain MPO mRNA and protein expression levels peaked at 1 and 2 d after MPTP exposure, respectively (Fig. 1*C*), which is contemporaneous to the most-intense phase of SNpc dopaminergic neuronal death in this PD model (Przedborski and Vila, 2001). We next asked whether the observed changes in MPO ventral midbrain content in MPTP-injected animals paralleled an alteration of MPO enzymatic activity by monitoring oxidation of tetramethylbenzidine. Consistent with the protein data, we found that ventral midbrain MPO activity also rose during MPTP neurotoxicity in a time-dependent manner (Fig. 1*D*). In contrast, in mutant mice deficient in MPO ($MPO^{-/-}$; $n = 2$), the ventral midbrain did not show higher oxidation of tetramethylbenzidine after MPTP administration (data not shown). Unlike in the ventral midbrain, levels of MPO mRNA, proteins, and catalytic activity in the cerebellum (brain region resistant to MPTP) were unaffected by MPTP administration. However, more unexpected was the finding that no MPO alteration could be detected in the striatum (where dopaminergic fibers degenerate after MPTP administration), as illustrated by the lack of change in striatal MPO activity: saline, 14.0 ± 4.1 ($n = 7$), versus MPTP (at 2 d), 16.2 ± 1.5 ($n = 11$; $p > 0.05$). Thus, both protein levels and activity of MPO increase in the MPTP mouse model of PD, specifically in ventral midbrain where the demise of the nigrostriatal dopaminergic neurons is taking place.

MPO is expressed in reactive astrocytes after MPTP injection

To elucidate the cellular origin of MPO in the ventral midbrain of MPTP-treated mice, immunohistochemical studies were performed. In saline controls, diffuse MPO immunoreactivity was seen in the neuropil (Fig. 2*A*,*C*). In MPTP-treated mice 2 d after the last injection, ventral midbrain MPO immunostaining was stronger, especially at the level of the substantia nigra, and cells with a glial morphology appeared labeled (Fig. 2*B*,*D*). These MPO-positive cells showed punctate immunoreactivity over both the cell bodies and proximal processes (Fig. 2*D*). To corroborate the bright-field microscopy results, we performed double-immunofluorescence confocal microscopy on ventral midbrain sections from mice 2 d after MPTP. This analysis confirmed that MPO colocalized with the astrocytic marker GFAP as shown by the merged image from the two fluorochromes (Fig. 2*E–G*) and

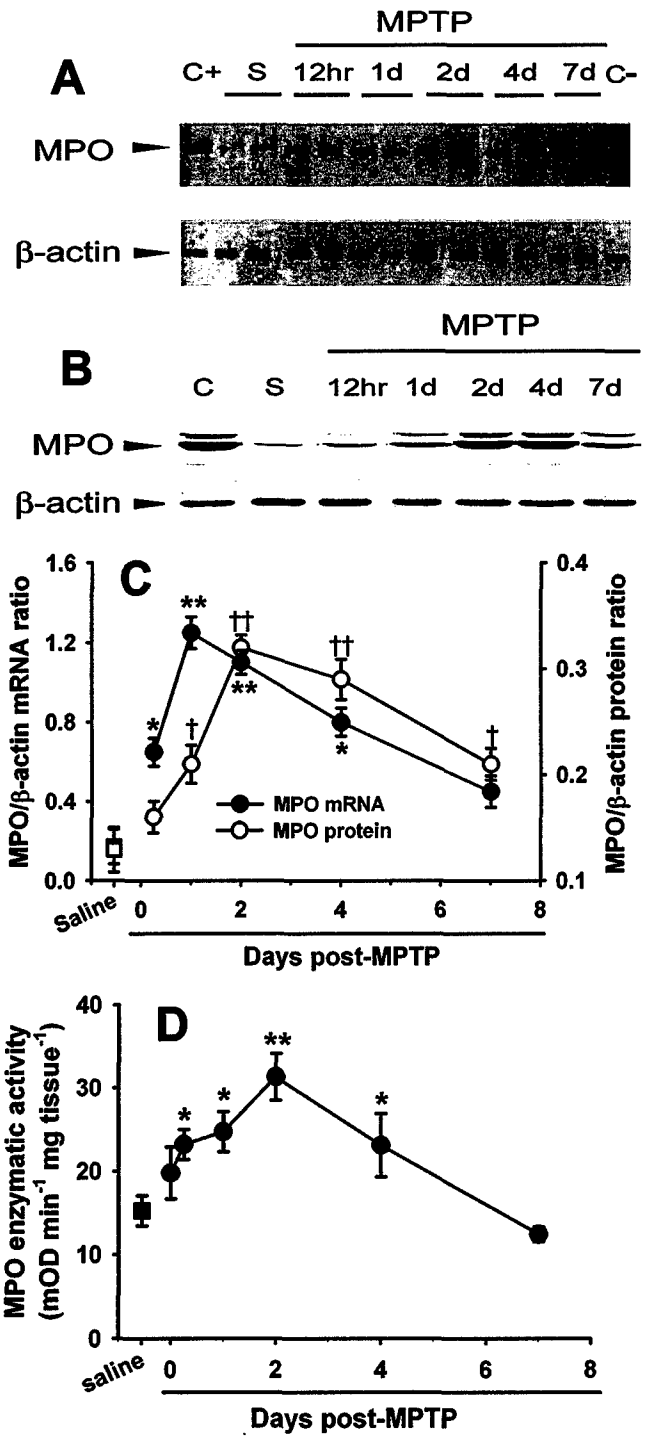

Figure 1. MPTP injections are associated with a time-dependent increase in ventral midbrain MPO mRNA (*A*, *C*), protein expression (*B*, *C*), and enzymatic activity (*D*) relative to saline injections. Data are means ± SEM for 3–11 mice per group. $^{*,\dagger}p < 0.05$, $^{**,\dagger\dagger}p < 0.01$ compared (Newman–Keuls *post hoc* test) with saline-injected control animals. S, Saline; C+, positive control (bone marrow); C−, negative control (absence of reverse transcriptase); mOD, millioptical density.

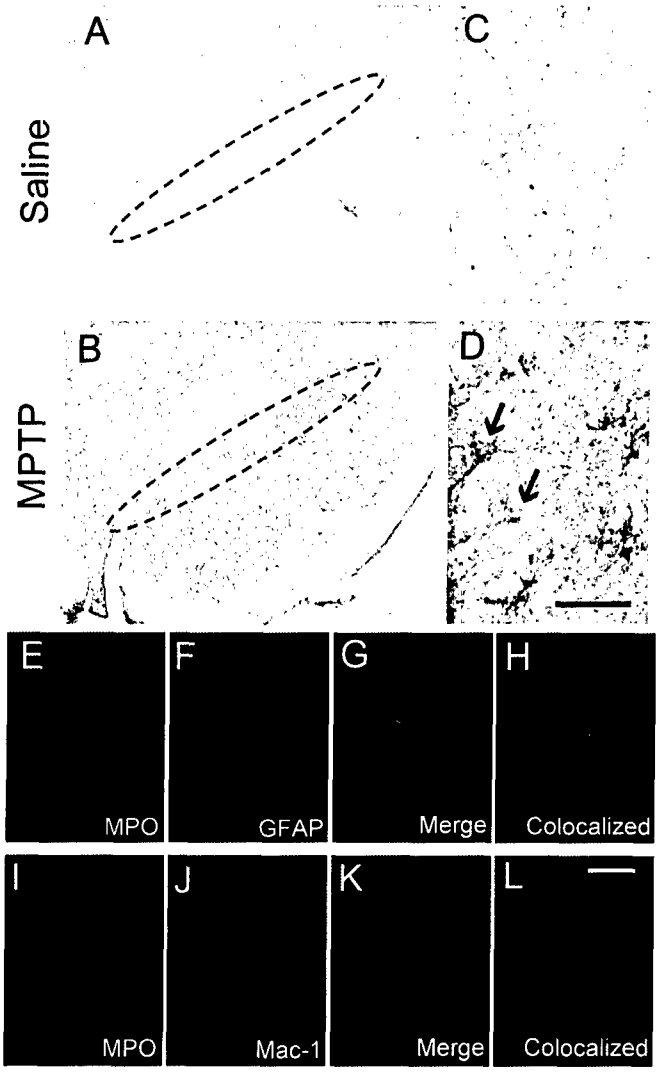

Figure 2. ***A, C***, Immunochemical studies revealed no specific MPO immunoreactivity in the ventral midbrain of saline-injected control mice. The dashed oval delineates the SNpc. ***B, D***, However, a dense network of fibers and scattered cell bodies positive for MPO are seen at the level of the SNpc after MPTP injections. Black arrows in ***D*** show the MPO-positive cellular elements. ***E–H***, Confocal microscopy demonstrates that ventral midbrain MPO-positive structures (***E***, red) are also GFAP positive (***F***, green), as evidenced by the overlay of the two fluorochromes (***G***) and by the computed mask of the colocalized pixels (***H***). ***J–L***, In contrast, ventral midbrain MPO-positive structures (***I***, red) are not MAC-1 positive (***J***, green), as evidenced by the overlay (***K***) and the mask of colocalized pixels (***L***). Tissue sections are from mice at 24 and 48 h after saline or MPTP injections. Scale bars: (in ***D***) ***A, B***, 250 μm; ***C, D***, 25 μm; (in ***L***) ***E–L***, 10 μm.

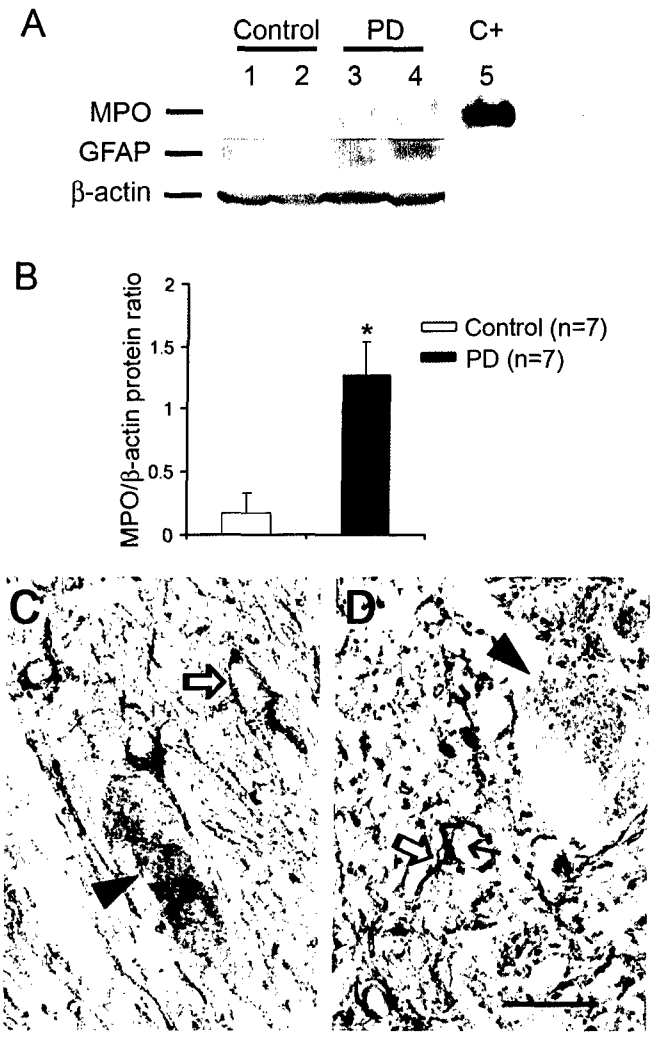

Figure 3. ***A, B***, Ventral midbrain MPO tissue content is increased in postmortem tissue from PD patients compared with controls, as well as GFAP tissue content. C+, Positive control (purified MPO). ***C***, In ventral midbrain sections, MPO (blue) is not detected in control tissues, neither in GFAP-positive cells (open arrow) nor in or around neuromelanized dopaminergic neurons (arrowhead). ***D***, Conversely, MPO immunoreactivity (blue, small black arrow) is found in GFAP-positive cells (open arrow) in PD tissue but not in the rare remaining neuromelanized dopaminergic neurons (arrowhead). Scale bar, 20 μm. Data are means ± SEM for seven samples per group. *$p < 0.05$ compared with normal controls (Newman–Keuls *post hoc* test).

the computed mask of the colocalized pixels (Fig. 2*H*). Conversely, no evidence of MPO expression in microglial cells could be documented by using the same techniques (Fig. 2*I–L*). Although abundant neutrophils were seen in our mouse bone marrow preparations (positive controls) using the anti-mouse neutrophil antibody MCA771GA, none were detected within the brain parenchyma (data not shown). No noticeable cellular MPO immunoreactivity was observed in the striatum or cerebellum of either saline- or MPTP-treated mice (data not shown). These results demonstrate that MPO is primarily expressed in ventral midbrain astrocytes during the demise of dopaminergic neurons caused by MPTP.

Expression of MPO is increased in PD midbrain

To determine whether the changes in MPO observed in the MPTP mouse model of PD were present in the human condition, we assessed MPO protein levels in postmortem ventral midbrain samples from sporadic PD patients. Consistent with the mouse data, PD samples had significantly higher MPO protein contents compared with controls (Fig. 3*A,B*). Like in mice, there was no significant difference in MPO to β-actin ratios in the striatum (PD, 1.1 ± 0.8, vs controls, 1.4 ± 0.8; $p > 0.05$; $n = 7$) or cerebellum (PD, 0.8 ± 0.2, vs controls, 1.0 ± 0.3; $p > 0.05$; $n = 7$) between the PD and control samples. Histologically, cellular MPO immunoreactivity was not detected in the control ventral midbrain parenchyma per se (Fig. 3*C*) but only in small cells within blood vessels. However, MPO immunoreactivity was seen in ventral midbrain sections from PD patients (Fig. 3*D*), where it was identified in SNpc glial cells in the vicinity of neuromelanin-containing neurons (Fig. 3*D*). The similarity of the MPO alterations between the MPTP mice and the PD postmortem specimens strengthens the relevance of using this experimental model to study the role of MPO in the PD neurodegenerative process.

Because gliosis is a common pathological feature of many neurodegenerative diseases, we wondered whether increases in

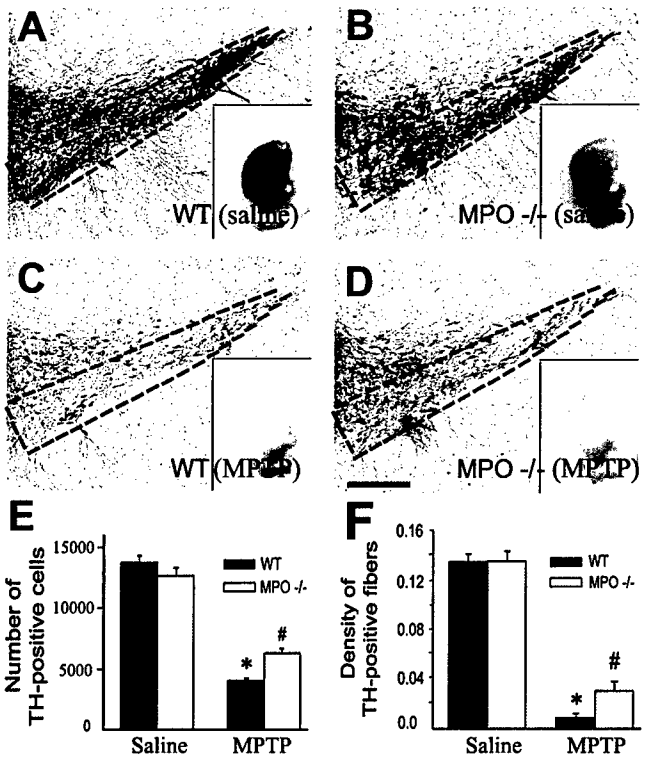

Figure 4. ***A–D***, Ablation of MPO in mutant mice attenuates MPTP-induced striatal TH fibers and SNpc TH neuronal loss, as assessed 7 d after either saline or MPTP injections. ***E***, ***F***, Quantification of neuronal (***E***) and fiber (***F***) loss. Data are means ± SEM for four to six mice per group. $^{*}p < 0.05$ compared with saline-injected animals; $^{\#}p < 0.05$ compared with saline- and MPTP-injected MPO $^{+/+}$ mice.

the expression of MPO within areas of neurodegeneration can be found in neurodegenerative disorders other than PD. Compared with controls, the motor cortex from ALS patients did not exhibit higher GFAP or MPO values (data not shown). Conversely, we found that caudate nucleus tissues from stage 4 HD patients had higher GFAP to β-actin ratios (HD, 0.7 ± 0.1, vs controls, 0.1 ± 0.1; $p < 0.01$; $n = 3–4$) as well as MPO to β-actin ratios (HD, 0.8 ± 0.2, vs controls, 0.2 ± 0.1; $p < 0.05$; $n = 5–6$). This suggests that brain MPO expression is not specific to PD but rather generic to neurodegenerative diseases in which areas of neuronal loss are accompanied with gliosis.

MPO deficiency protects against MPTP-induced neurodegeneration

Next we compared the effects of MPTP on the nigrostriatal pathway of mutant mice deficient in MPO (MPO $^{-/-}$) and their WT littermates (MPO $^{+/+}$). Seven days after the last injection of saline or MPTP, the brains of these animals were processed for quantification of dopaminergic cell bodies in the SNpc and of projecting dopaminergic fibers in the striatum using TH immunostaining. In saline-injected MPO $^{-/-}$ and MPO $^{+/+}$ mice, stereological counts of SNpc dopaminergic neurons and striatal TH-positive OD (Fig. 4*A*,*B*,*E*,*F*) were comparable. In MPTP-injected MPO $^{+/+}$ mice, there was a ~70% loss of SNpc TH-positive neurons (Fig. 4*C*,*E*) and ~92% reduction of striatal TH OD (Fig. 4*C*,*F*) compared with saline-injected controls (Fig. 4*A*,*E*,*F*). In contrast, in MPTP-injected MPO $^{-/-}$ mice, there was only ~50% loss of SNpc TH-positive neurons (Fig. 4*D*,*E*) and ~70% reduction of striatal TH OD (Fig. 4*D*,*F*) compared with saline-injected controls (Fig. 4*B*,*E*,*F*). The TH/Nissl ratio of neuronal counts did not differ between saline- and MPTP-injected WT mice (saline, 1.68 ± 0.15, vs MPTP, 1.46 ± 0.20; $n = 5$ per group), supporting the assertion that the reduction in dopaminergic neuron numbers corresponds to an actual cell loss and not to a downregulation of TH.

To examine whether MPO ablation protects not only against structural damage but also against functional deficits caused by MPTP, we compared the levels of dopamine and its metabolites, dihydroxyphenylacetic acid and homovanillic acid, in the striatum as well as locomotor activity between MPO $^{-/-}$ and MPO $^{+/+}$ mice, after MPTP injections. Contrasting with the protection afforded by the lack of MPO on the nigrostriatal dopaminergic neurons, the loss of striatal dopamine and the deficit in motor performance caused by MPTP were as severe in MPO $^{-/-}$ as in MPO $^{+/+}$ mice (supplemental material, available at www.jneurosci.org).

MPTP metabolism

Major factors controlling MPTP neurotoxicity are its conversion in the brain to MPP $^{+}$, followed by MPP $^{+}$ entry into dopaminergic neurons and its subsequent blockade of mitochondrial respiration (Przedborski and Vila, 2001). To ascertain that the resistance of MPO $^{-/-}$ mice was not attributable to alterations in MPTP toxicokinetics, we assessed its three key neurotoxic steps (Tieu et al., 2003). Results show that striatal levels of MPP $^{+}$, striatal uptake of [^{3}H]MPP $^{+}$, and MPP $^{+}$-induced lactate production (a measure of mitochondrial function) did not differ between MPO $^{-/-}$ mice and their WT littermates (Table 1).

MPO damages ventral midbrain proteins

MPO is the only known mammalian source of HOCl at plasma concentrations of halide ion (Gaut et al., 2001). HOCl reacts with tyrosine to form 3-chlorotyrosine, a specific and stable biomarker of protein damage by MPO (Heinecke et al., 1999). To determine whether MPTP promotes oxidative damage to brain proteins, we used isotope dilution GC/MS (Heinecke et al., 1999), a sensitive and specific method, to quantify 3-chlorotyrosine levels in samples from eight saline-injected controls and eight MPTP-injected mice 24 h after injection. We compared levels of 3-chlorotyrosine in the ventral midbrain and cerebellum. In MPTP-treated mice, 3-chlorotyrosine levels in the ventral midbrain were markedly increased ($p < 0.05$) compared with saline-injected controls: MPTP, 30.8 ± 5.7 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$) versus saline controls, 4.8 ± 2.1 nmol of 3-chlorotyrosine per molar of tyrosine ($n = 8$). 3-Chlorotyrosine was undetectable in the cerebellum of mice injected with either saline or MPTP. In contrast, in MPTP-treated MPO $^{-/-}$ mice ($n = 3$), ventral midbrain 3-chlorotyrosine was undetectable. The identification of chlorinated tyrosine in tissues therefore supports the hypothesis that reactive intermediates produced by MPO damage brain proteins in MPTP-intoxicated mice.

To localize MPO-damaged proteins, tissue sections were immunostained with HOP-1, a mouse antibody that specifically recognizes HOCl-modified proteins (Malle et al., 1995); the chlorotyrosine antibody was not available to us. Intense HOP-1 immunoreactivity was observed in the SNpc of MPTP-injected mice (Fig. 5*A–C*). HOP-1-positive material was seen in the neuropil within beaded-appearing fibers and in cells with both neuronal and non-neuronal morphology within vesicular elements (Fig. 5*A–C*). No HOP-1 immunostaining was detected in the SNpc of saline-injected mice or MPTP-injected MPO $^{-/-}$ mice (data not shown).

Table 1. Striatal MPTP metabolism in MPO-deficient mice

	MPP^+ level (μg/g striatum)	MPP^+ uptake (IC_{50}, nM)	MPP^+-induced lactate (μM/100 mg protein)
$MPO^{+/+}$ mice	4.46 ± 0.24	113.7 ± 1.2	57.6 ± 7.5
$MPO^{-/-}$ mice	5.54 ± 0.71	114.3 ± 1.7	66.8 ± 4.4

Striatal MPP^+ levels in WT ($MPO^{+/+}$) and MPO-deficient mice ($MPO^{-/-}$) were determined 90 min after the last injection of MPTP (20 mg/kg). Values are means ± SEM of either six mice per group (MPP^+ level) or three independent experiments each performed in duplicate ([^{3}H]MPP^+ uptake and lactate level). None of the presented values differ significantly ($p > 0.05$) between $MPO^{+/+}$ and $MPO^{-/-}$ mice.

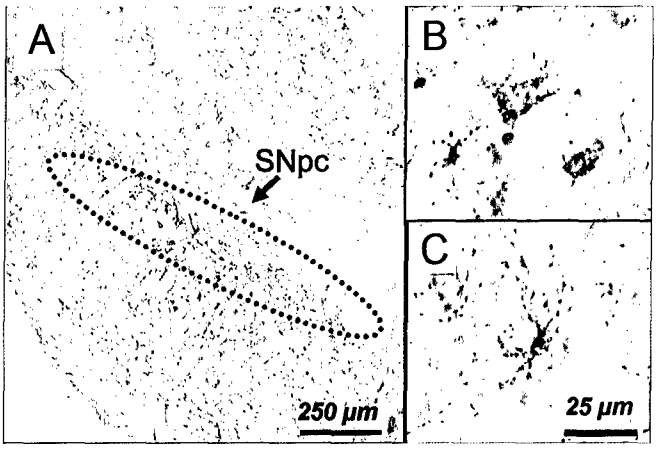

Figure 5. Immunohistochemical localization of HOCl-modified proteins with the HOP-1 antibody in ventral midbrain sections. Twenty-four hours after MPTP injections, HOP-1-positive immunoreactive material is seen mainly at the level of the SNpc (***A***) and within or around cellular elements (***B***, ***C***). Scale bars: ***A***, 250 μm; ***B***, ***C***, 25 μm.

Discussion

The present study shows that the level of MPO expression increases markedly in diseased SNpc from both mice exposed to MPTP (Figs. 1, 2) and human PD (Fig. 3). This work also demonstrates that changes of MPO protein content and enzymatic activity in MPTP-intoxicated mice parallel (Fig. 1) the degeneration of SNpc dopaminergic neurons (Przedborski and Vila, 2001). Moreover, MPO is found primarily in SNpc-reactive astrocytes (Figs. 2, 3), which are major cellular components of the PD- and MPTP-associated inflammatory response (Przedborski and Goldman, 2004). Conversely, we failed to detect any of the well established cellular sources of MPO (neutrophils, monocytes, or macrophages) (Hampton et al., 1998) within the ventral midbrain parenchyma of PD patients and MPTP-injected mice. The presence of MPO in damaged SNpc thus appears to derive essentially from a resident, not a blood-borne, inflammatory response associated with the degeneration of dopaminergic neurons. Based on assessments performed in two other neurodegenerative diseases, namely HD and ALS, it appears that MPO upregulation in the brain is not pathognomonic of PD. Instead, we believe that the occurrence of MPO in diseased brains is likely indicative of a disease process associated with chronic gliosis rather than a particular etiology. That said, our results are surprising because phagocytic white blood cells are generally believed to be the only cellular sources of MPO. However, neuronal expression of MPO is also increased in Alzheimer's disease (Green et al., 2004), raising the possibility that this enzyme may contribute to oxidative damage in a variety of chronic neurodegenerative disorders.

Contrary to ventral midbrain, the striatum, which is also a site of a strong inflammatory reaction after MPTP administration and sometimes in PD, did not show any alteration in MPO expression or enzymatic activity as illustrated in Figure 3. Remarkably, detectable changes in iNOS expression and enzymatic activity are also confined to ventral midbrains of MPTP-injected mice (Liberatore et al., 1999), whereas activation of NADPH oxidase is observed in both ventral midbrains and striata of these animals (Wu et al., 2003). Collectively, these observations suggest that the molecular composition of the inflammatory response to injury may be, to a certain extent, regionally specific. Supporting this view is the finding that a stereotaxic injection of 5 μg/μl bacterial endotoxin lipopolysaccharide into the hippocampus or cortex of adult rats produces no apparent neuronal loss, whereas an identical administration into the substantia nigra dramatically reduces the number of neurons (Kim et al., 2000). Although this distinct regional susceptibility has been linked to differences in microglial densities, its molecular basis might well be related to differences in the quantity or variety of the inflammatory mediators produced.

After MPTP injections, mutant mice deficient in MPO showed more spared SNpc dopaminergic neurons and striatal dopaminergic fibers than their WT littermates (Fig. 4). We also found that the lack of MPO did not alter key aspects of MPTP toxicokinetics (Table 1). Together, these findings indicate that MPO contributes to the pathogenic cascade of deleterious events responsible for the demise of dopaminergic neurons in the MPTP model and perhaps in PD as well. Surprisingly, although alterations in MPO protein and enzymatic activity were only detected in the ventral midbrain (Fig. 1), both cell bodies and fibers of nigrostriatal dopaminergic neurons were preserved in MPTP-injected $MPO^{-/-}$ mice (Fig. 4). This observation implies that an entire neuron may be salvaged by mitigating deleterious factors that specifically injure cell bodies and that nigrostriatal dopaminergic neurons are not degenerating solely via a dying-back process, as one may have thought based on previous observations (Herkenham et al., 1991; Wu et al., 2003).

The relative resistance of dopaminergic neurons to MPTP-induced neurotoxicity in $MPO^{-/-}$ mice was, however, not accompanied by a preservation of striatal dopamine levels or attenuation of motor deficits caused by this parkinsonian neurotoxin (supplemental material, available at www.jneurosci.org). This discrepancy may be explained by the fact that TH (the rate-limiting enzyme in the synthesis of dopamine) can be inactivated by injury, such as that inflicted by MPTP (Ara et al., 1998). It is thus conceivable that although ablation of MPO attenuates the loss of TH protein (as evidenced by immunostaining), this beneficial effect may not be enough to prevent the loss of TH catalytic activity (as evidenced by the dopamine levels). Targeting MPO alone may thus suffice to provide observable structural, but not functional, neuroprotection in this experimental model of PD. Accordingly, optimal therapeutic interventions for PD may rely on the combination of strategies capable of providing structural protection such as MPO inhibition, with other strategies capable of protecting/stimulating dopaminergic function. Yet, given the relentless nature of PD, it can be surmised that the death signal in this illness may not be as harsh as that provoked by MPTP. Therefore, whether MPO inhibition in PD can succeed, not only in slowing neuronal death but also in sustaining dopamine synthesis, is a possibility that should not readily be excluded.

As to how MPO neurotoxic actions on dopaminergic neurons are mediated, two distinct and not mutually exclusive mechanisms may be invoked. First and foremost, MPO is known for its production of cytotoxic reactive oxygen species and RNS (Harrison and Schultz, 1976; Eiserich et al., 1996; Hampton et al., 1998).

Therefore, neurons located in the vicinity of MPO-containing cells may have their plasma membrane proteins and lipids subjected to the deleterious effects of MPO-derived oxidants such as HOCl. In keeping with this scenario, we found high levels of 3-chlorotyrosine, a specific oxidative modification of tyrosine residues mediated by HOCl in the MPTP-susceptible brain region, the ventral midbrain. Also supporting the oxidative role of MPO in the MPTP model is our immunohistochemical demonstration of HOCl-modified protein in the ventral midbrain of intoxicated mice (Fig. 5*A–C*). Aside from this oxidative effect, MPO can be secreted and bind CD11b/CD18 integrins to the cell surface (Lau et al., 2005). In the case of neutrophils, ligation of CD11b/CD18 by MPO stimulates signaling pathways implicated in the activation of these cells (Lau et al., 2005). Because brain microglia do express CD11b/CD18 integrins and seem to participate in the neurodegenerative process in the MPTP model and in PD, this cytokine-like effect of MPO may represent an additional mechanism by which dopaminergic neurons are affected by this enzyme.

As raised previously (Wu et al., 2003), a key issue is the selective damage to dopaminergic neurons observed during inflammation in MPTP-treated mice and humans suffering from PD. Many lines of evidence suggest that dopaminergic neurons are particularly vulnerable to oxidative stress compared with the other cells in the brain (Dauer and Przedborski, 2003). Alternatively, it is likely that in the MPTP model and in PD, the magnitude of the inflammatory response and resulting oxidative stress is mild and only inflicts sublethal lesions. Thus, inflammation-mediated oxidative stress would succeed in killing only neurons already compromised, as dopaminergic neurons probably are in PD and after MPTP injections.

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eNOS Figures and Tables

FIG.1. The expression in of eNOS mRNA ventral midbrain samples of saline-treated (S) and MPTP-intoxicated mice at different time points after intoxication. The Ethidium bromide signals of RT-PCR for eNOS result in a single band of 354 bp (A). For quantification, The radioactive RT-PCR was used (panel B) for detection of eNOS and internal control (GAPDH) mRNA levels. The bar graph (C) shows the ratio of eNOS/GAPDH optical density from the midbrains of saline and 0, 2, 4, 7days after MPTP injection. Every time points after MPTP injection is not different compared with saline ($p>0.05$); n=5-6 for each group.

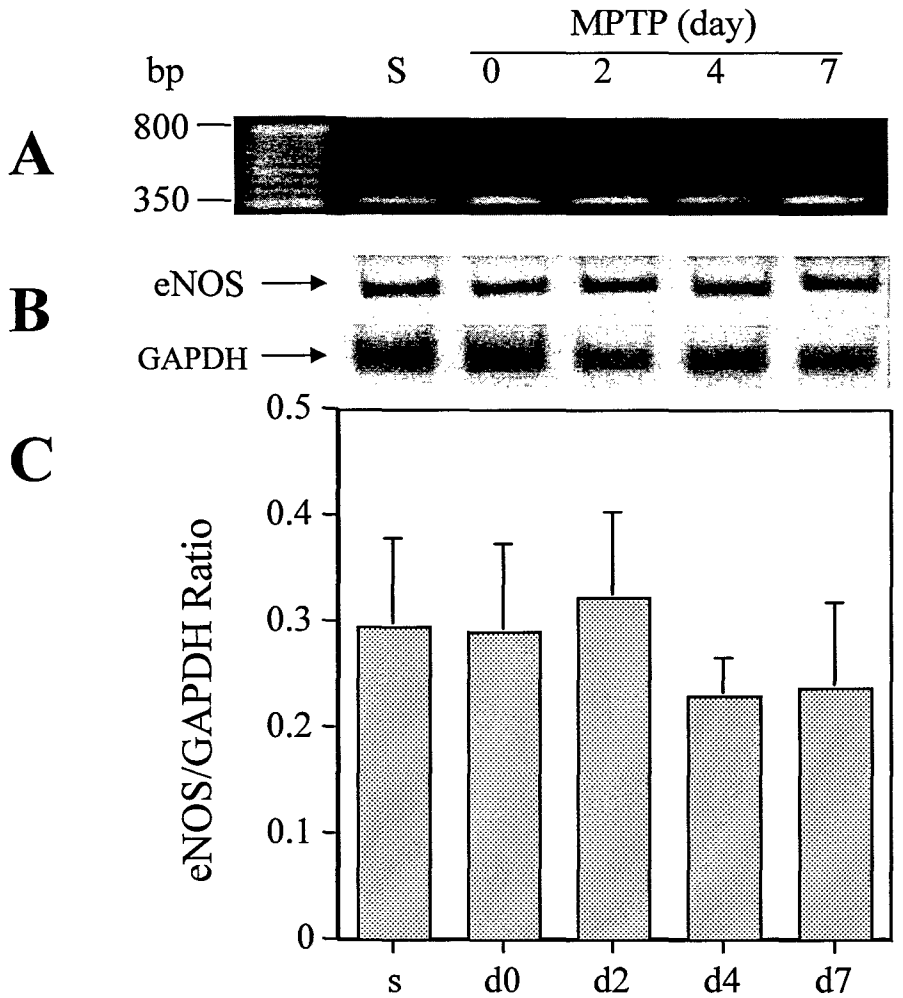
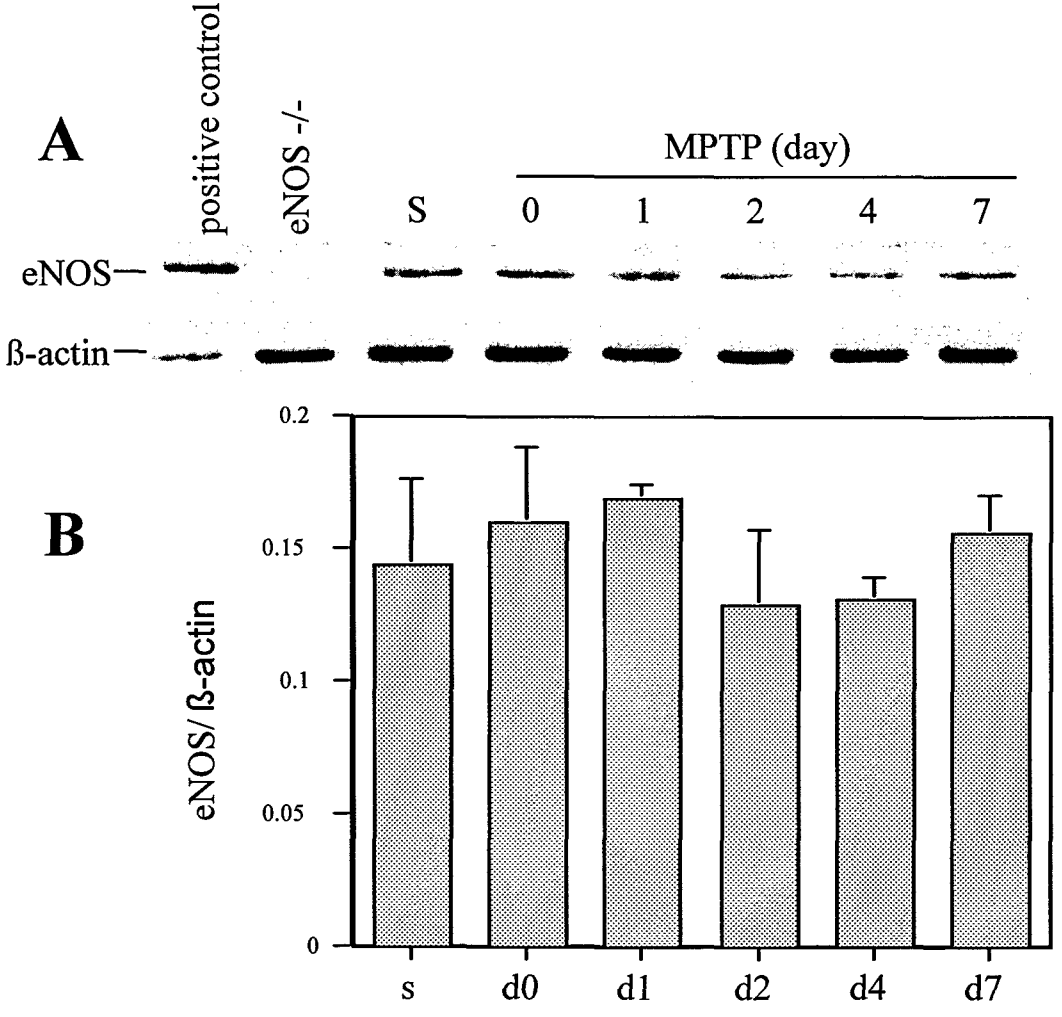


FIG. 2. Expression of eNOS in ventral midbrain samples of saline-treated (S) and MPTP-treated mice at different time points after intoxication. The midbrain sample from eNOS -/- mice was used for negative control. The human endothelial was used for positive control. 135 kDa eNOS band is indicated in panel A. Quatitative results were obtained by measurement of the optical density of each band using a computarized image analysis system as described in Material and Methods. The bar graph (B) show ratio of eNOS/ß-actin. There are no changes of eNOS protein levels in midbrains of saline-treated and MPTP-treated mice ($p>0.05$).



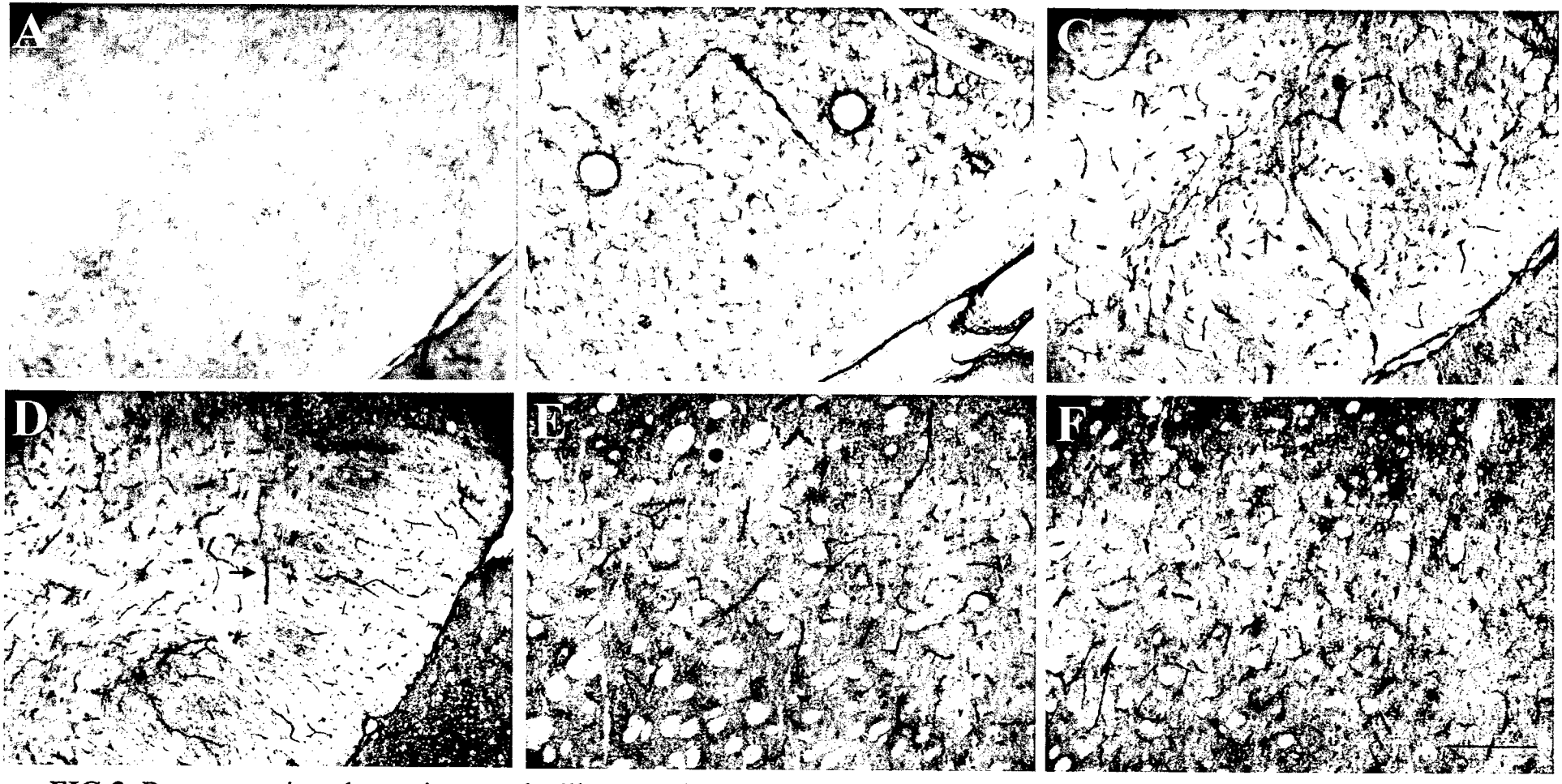


FIG.3. Representative photomicrographs illustrate the eNOS immunoreactivity in the ventral midbrain. There is no dark black staining in the negative control sections (A) and a little eNOS immunoreactivity in the eNOS-/- midbrain section (B). There are high and related homogenized eNOS immunostaining in the midbrain of saline-injection mice(C). No alteration was obseved at the levels of eNOS-positive blood vessels in the substantia nigra of MPTP-treated mice (D). There are also no difference in the striata of saline-injected (E) and MPTP-treated (F) mice. Scale bar=200 μm.

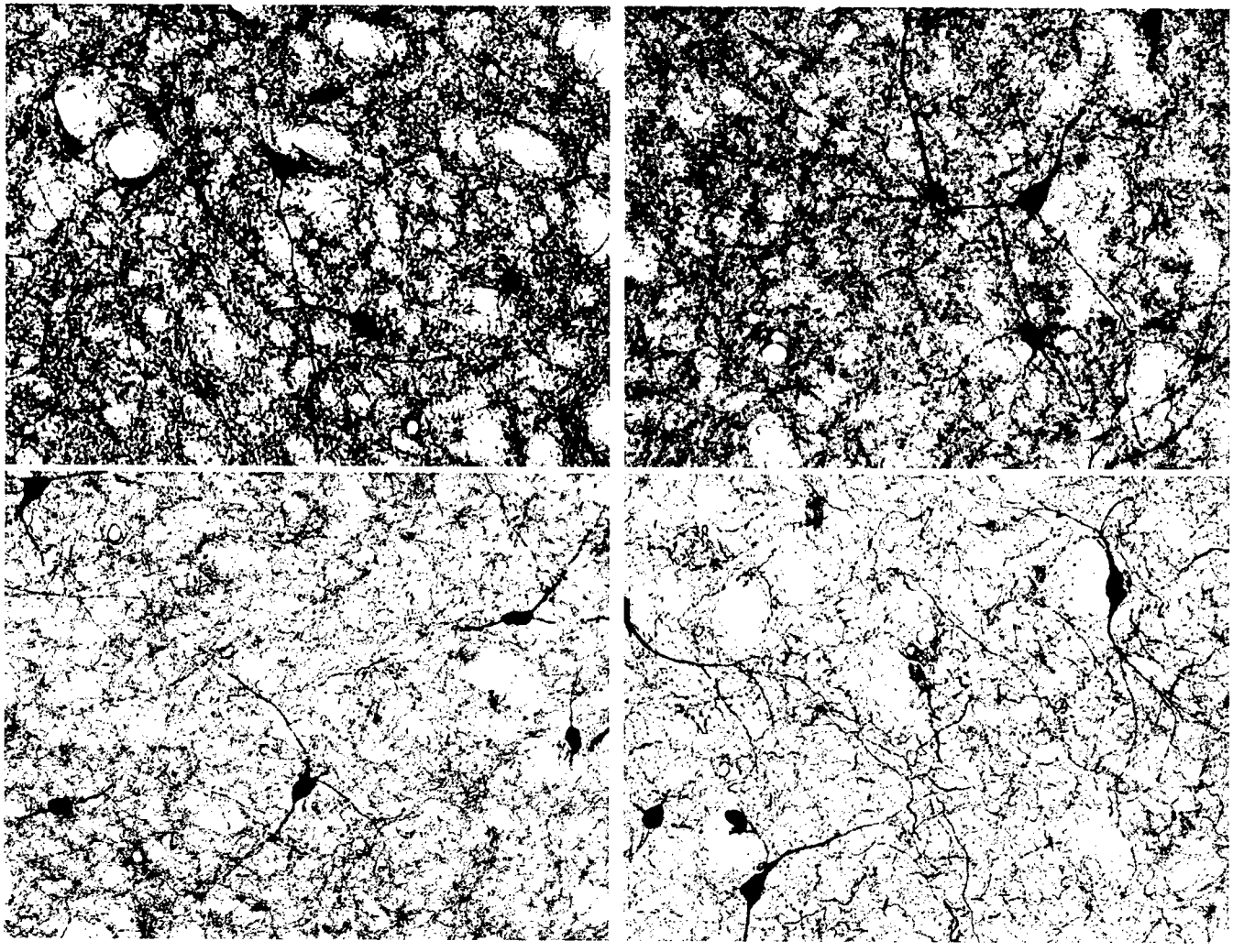


FIG.5. Representative photomicrographs illustrate the NADPH activity (blue) and TH fiber (brown) immunoreactivity in the striatum. There is no blood vessel-like blue staining in the eNOS-/- midbrain section (A). There are only a few blood vessel-like blue staining blue in striatum of saline-injection mice(B), MPTP-treated mice at 1 day after injection(C) and 7 days after injection (D). Scale bar=50 μm.

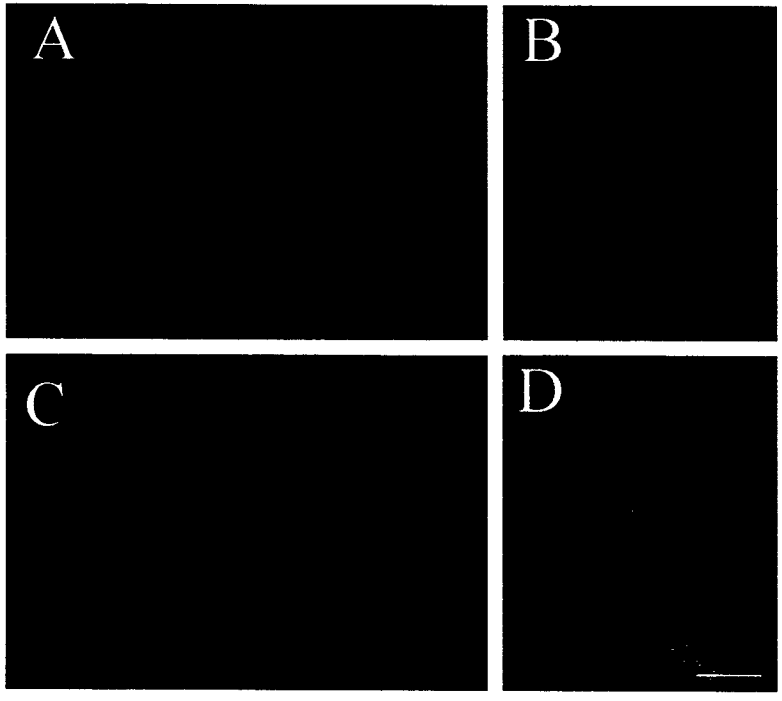


Fig.6. Representative photomicrographs illustrate the Lycopersicon esculentum lectin fluorescein isothiocyanate staining. There are high and related homogenized vasculature staining in the midbrain of mice(A, B) and in the striatum (C, D). Scale bar=100 μm (A, C); 10 μm (B, D).

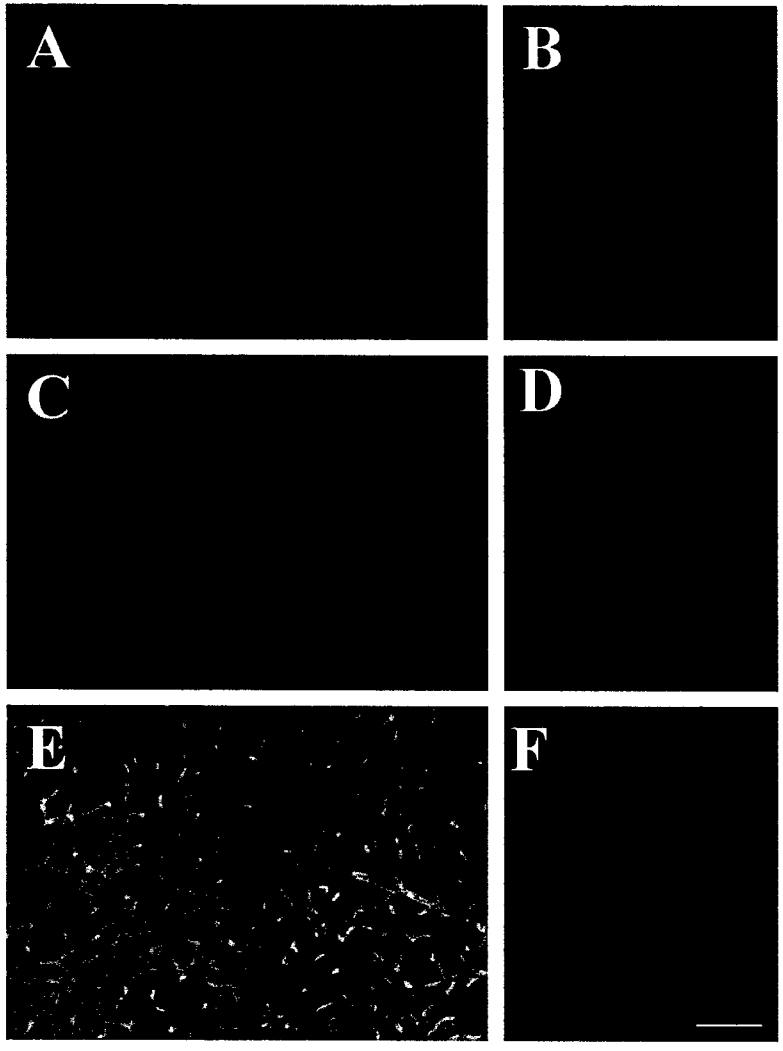


FIG.7. Representative photomicrographs illustrate the Lycopersicon esculentum lectin fluorescein isothiocyanate staining (A, B, E, F) and TH staining (C, D, E, F). There are high and related homogenized vasculature staining in the midbrain of mice (A). Scale bar=100 μm (A, C, E); 10 μm (B, D, F).

Table 1 MPP^+ levels (µg/g striatum)				
	5 min	30 min	90 min	240 min
Wild type	2.31 ± 0.12	8.55 ± 2.05	9.83 ± 2.12	3.69 ± 0.26
eNOS -/-	2.59 ± 0.66	7.02 ± 0.87	7.70 ± 2.44	2.69 ± 0.27

Striatal MPP^+ levels in wild-type and eNOS -/- mice at 5, 30, 90, 240 min after the last MPTP injection do not differ ($p > 0.05$; Newman-Keuls post-hoc test) between the two groups. Data represent means ± s.e.m. for three to four mice per group and time point.

Table 2 Number of neurons in the SNPc

	Saline		MPTP	
	Wild-type	eNOS -/-	Wild-type	eNOS -/-
Tyrosine hydroxylase	8554 ± 293	8390 ± 467	4229 ± 618*	3743 ± 273**
Number of Neurons	17900 ± 798	17294 ± 649	12261 ± 648*	11833 ± 632**

SNPc neurons (mean ± s.e.m.; n=4-6 per group) were counted by sterology, *, $p < 0.05$, fewer than both saline groups; **, $p < 0.05$, fewer than both saline-injected groups and non different with MPTP wild-type mice; Newman-Keuls post-hoc test.